INTRODUCTION

1. The Eph-ephrin system

1.1 Structure and signal activation

The Eph receptors (erythropoietin-producing hepatocellular carcinoma) have been originally identified more than two decades ago, but new aspects of these proteins come out constantly.

They represent the largest family of receptor tyrosine kinases (RTKs) and they are subdivided into class A and class B based on sequence homology and binding affinity for two distinct types of membrane-anchored ligands called ephrins (Eph receptor interacting proteins) .

EphA and EphB receptors have a similar modular structure [Fig. 1]: they contain an extracellular region, which includes a highly conserved N-terminal domain, a cysteine rich-region and two fibronectin-type III repeats, and an intracellular side with a iuxtamembran region, a conserved kinase domain, a SAM (sterile-α-motif) domain and a PDZ domain-binding motif [Himanen et al., 2009]. The ligand binding domain (LBD), situated in the amino terminal side of the extracellular domain contains an high affinity binding site that mediates receptor-ephrin interaction between cells [Pasquale, 2008; Himanen et al., 2007], but also two additional low-affinity sites have been identified in LBD and cysteine domain, which are important for the formation and stabilization of clustered Eph receptor-ephrin complexes [Pasquale, 2005].

The ephrin ligands are also subdivided into A and B classes. In humans, five glycosyl-phosphatidyl-inositole (GPI)-linked ephrin-A ligands bind nine EphA receptors and three transmembrane ephrin-B ligands bind five EphB receptors [Fig. 1]. The A class receptors preferentially bind A-type ligands, while the B class receptors bind B-type ligands, but there are some exceptions: EphA4 can bind both A-type and B-type ephrins, while ephrin-A5 binds EphB2 besides the EphA type, but not other EphB receptors.

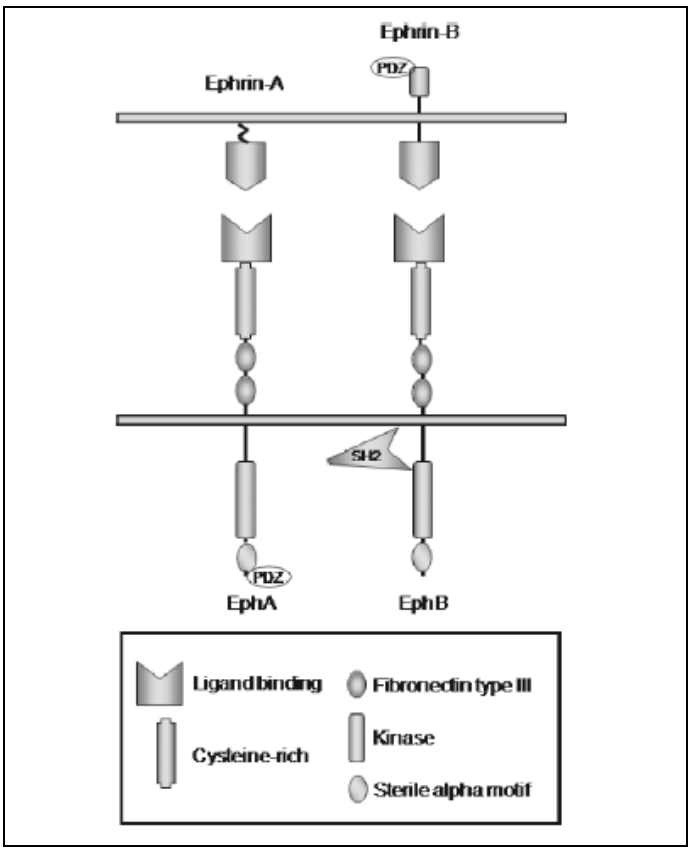


Figure 1: Eph-ephrin representative structure. Eph receptors have similar modular structure characterized by a ligand binding domain, a cysteine rich region, two fibronectin III repeats and a intracellular kinase domain. Ephrin ligands are membrane anchored: ephrin-A ligands through a GPI link while ephrin-B ligands are transmembrane proteins [Ahmed et al., 2009].

Eph receptor signaling can be activated after cell-cell contact and then transmitted bidirectionally: not only the receptor-expressing cell can transduce a signal (forward signal), but even the ligand-expressing cell can generate it (reverse signal). Additionally, both Eph receptors and ephrin ligands are able to expand signals independently of their interaction, through crosstalk with other pathways [Pasquale, 2010].

Eph-ephrin signals can be generated with formation of molecular complexes. First of all, a monovalent interaction between receptor and ligand, due to the progressive cell-cell contact, starts assemblage of the cluster: ephrin ligand protrudes its hydrophobic loop into the Eph receptor cavity in a high-affinity interface forming a receptor-ligand heterodimer. Subsequently, Eph-ephrin binding in the lower-affinity interfaces can mediate a further dimerization generating a tetramer, which can progressively aggregate into large clusters based on the number and density of Eph receptors and ephrins on the cell surface [Pasquale, 2005].

The assembled tetramer leads to the proximity of two receptor cytoplasmic domains that phosphorylate each other on tyrosine residues, changing the conformation of the iuxtamembrane domain and promoting the signal cascade. Ephrin-B ligands are also phosphorylated on tyrosine residues in the cytoplasmic domain after interaction with Src-kinases, generating the reverse signal.

The shutdown of the signal is mediated by recruitment of phosphatases, which dephosphorylated the tyrosine in kinase domain, and adaptor proteins like c-Cbl responsible for receptor internalization and degradation. Also the metalloprotease ADAM 10 is involved in termination of the signal enhancing levels of ligand cleavage and endocytosis of the ligand-receptor complex [Salaita et al, 2010; Pasquale, 2005]. Interestingly, ADAM 10 has a cysteine rich domain that is able to recognize specifically ephrins bound to Ephs, so it can interact and consequently cleave only the ephrin in complex with the receptor.

1.2. Crosstalk with other pathways

Eph-ephrin signaling is extremely complex and often conflicting: bidirectional signaling often activates downstream signals with opposite effects. Further studies have to be conducted in order to clarify the role of this system in physiology and diseases. It is clear that the Eph-ephrin system can act in concert with other signaling pathways in the cell. Recent studies show interactions with EGFR (Epidermal Growth Factor Receptor): the activation of this receptor by EGF (Epidermal Growth Factor) induce EphA2 expression in mammalian cells [Larsen et al., 2010]. This cooperation

between EphA2 and EGFR can promote cell motility and proliferation independently of ephrin stimulation [Pasquale, 2008; Brantley-Sieders et al., 2008; Larsen et al., 2007].

E-cadherin can also regulate Eph receptor expression and localization on the cell surface: immunoistochemical studies show that E-cadherin downregulation reduces EphA2 arrangement on the cell membrane preventing clustering between Eph-ephrin-expressing cells [Arvanitis et al., 2008; Ireton et al., 2005; Herath et al., 2010; Zantek et al., 1999].

Moreover, recent evidence shows an interaction with Ras and MAPK (Mitogen Activated Protein Kinases): EphA2 controls Ras activity in a ligand-dependent manner by a negative feedback loop [Macrae et al., 2005; Menges et al., 2008] that inactivates the MAPK pathway and at the same time, the MAPK pathway suppresses ephrin-A1 expression and upregulates EphA2 levels.

It has also been shown that EphB2 positively regulates cell proliferation via an Abl kinase-cyclin D1 pathway and cell migration through PI3K (Phosphatidyl Inositol 3' Kinase) signaling [Genander et al., 2009].

Recently a regulatory loop between EphA2 and Akt has been described by Miao and colleagues: Akt is able to phosphorylate the EphA2 serine residue S 897 and this process can promote cell migration in a ligand-independent manner [Miao et al., 2009].

Lastly, it was shown that Eph kinases are able to mediate some cellular functions, such as adhesion/repulsion, by negatively regulating integrins and FAK (Focal Adhesion Kinases): when endogenous EphA2 is activated, integrins are led to an inactive conformation inhibiting integrin-mediate adhesion and also cell spreading and migration. Furthermore, EphA2 activation by the ephrin-A1 ligand immediately recruits SHP2, a protein tyrosine phosphatase that dephosphorylates FAK [Miao et al., 2000].

1.3 Cellular functions

Eph signaling culminates in modifications of actin cytoskeleton organization and microtubular dynamics, as well as regulation of intracellular and surface adhesion molecules, so it is involved in cell attraction/repulsion, migration, invasion, and morphology changes.

The bidirectional signals can activate an intricate cascade of pathways that often lead to opposite effects, and this can explain the different behavior of cells after the activation of the signal [Pasquale, 2008].

The importance of the Eph-ephrin system in adhesive/repulsive effects has been extensively studied and many reports have shown that alterations in the Eph-ephrin system could increase mortality: ephrin-A5 knockout mice have severe congenital malformations in the craniofacial and central nervous system area [Holmberg et al., 2000], while ephrin-A1 knockout mice have cardiac valve anomalies [Frieden et al., 2010]. In addition, mutations in ephrin-B2 or EphB2 and EphB3 lead to anorectal malformations [Dravis et al., 2004].

High affinity interactions between Eph receptors and ephrins in adjacent cells can also mediate cell-cell adhesion. After the binding with the ephrin ligands, Eph receptors are phosphorylated and initiate signals that regulates the balance between cell repulsion and adhesion. In general, forward signaling seems to be more often repulsive, while reverse signaling (mostly through ephrin-As) mediates preferentially adhesive effects, but exceptions have been found [Halloran et al., 2006].

The separation of two cells that adhere to each other through Eph-ephrin contact is a process where different mechanisms are involved. First, endocytosis and internalization of Eph-ephrin complexes plays an important role: several findings show that endocytosis is blocked and cells adhere strongly promoting adhesion when ephrin or Eph cytoplasmic domains are removed. Another important mechanism that can shift the balance between repulsion and adhesion/attraction is the proteolitic cleavage of the ephrins by the metalloprotease ADAM 10, which is able to cleave the extracellular domain of ephrins and whose activity is triggered specifically by receptor-ligand binding complex [Hattori et al., 2000]. Furthermore, a regulatory feedback mechanism generated by Eph signaling can inactivate adhesion molecules like E-cadherins enhancing cell-cell or cell-ECM (extracellular matrix) separation [Pasquale , 2005].

On the other hand, deficiencies in the activation of signaling pathways for repulsion can promote adhesion, leading to a shift in the cellular response from repulsion to adhesion. Phosphatases can be also recruited to shutdown the signal before cells have separated [Pasquale 2008; Palmer et al., 2002; Brantley-Sieders et al., 2004].

Interestingly, other factors such as the degree of clustering, which increases the signaling levels, and the time course lead the cell response in one direction mediating the switching balance between adhesion or repulsion.

Eph signals can also control cell morphological changes in different tissues by activating Rho proteins, GTPases involved in actomyosin contractility and remodeling of the actin cytoskeleton [Salaita et al., 2010; Surawska et al., 2004]. For instance, EphA4 stimulation activates Rho through GEFs (Guanine nucleotide Exchange Factors) leading to the retraction of retinal ganglion cell cone [Lawrenson et al., 2002], while the activation of EphA2 by ephrin-A1 induces prostate carcinoma cells rounding and increases their migration through the Rho-dependent actomyosin contractility response [Parri et al., 2007].

Different important biological processes, such as embryogenesis or angiogenesis, require an increase in cell migration for the correct compartmentalization of tissues during development and the correct cell localization in specific regions in the embryo [Pasquale, 2008; Wilkinson et al., 2001]. Both forward and reverse signaling are involved in cell migration and they can regulate several pathways including Rho proteins, integrin, FAK and MAPK to shift the balance between adhesion and repulsion [Murai et al., 2003; Kullander at al., 2002].

1.4 Eph-ephrin system in physiology

The Eph-ephrin system has been widely studied in embryogenesis, where it plays an important role in tissue compartmentalization and boundary development, in particular in the central nervous system. However, even in the adult Eph-ephrin bidirectional signaling modulates a variety of biological activities, including platelet aggregation, bone and intestinal homeostasis, immune system function, inflammation, glucose balance, angiogenesis.

1.4.1 Embryogenesis

Many reports have highlighted the key role of the Eph-ephrin system in embryogenesis. Activation of receptor-ligand complexes controls directional movements of cells and neuronal growth cones establishing the correct body plan in

the embryo. In developing tissues, different areas where Eph receptors are expressed are adjacent to areas where ephrin ligands are expressed: receptor distribution is often complementary and reciprocal to the distribution of their cognate ligands in embryonic tissues. This feature contributes to the interactions between adjacent populations of cells and subsequently it leads to their compartmentalization in a specific assembly through repulsive and adhesive processes [Dodelet et al., 2000; Gale et al., 1997].

The most important role of the Eph-ephrin system is during synaptogenesis, guiding axons to their targets and regulating neuronal connectivity. Several reports have shown that EphB receptors and ephrin-B ligands are involved in excitatory synapse development regulation, especially in hippocampal and cortical areas.

Interestingly, different Eph receptor domains are involved in controlling different aspects of synaptogenesis and the activation of a single portion is sufficient to promote the assembly of presynaptic structures [Pasquale, 2008; Kayser et al., 2006]. Eph receptors are able also to regulate dendritic spine morphology by interacting with actin cytoskeleton [Hruska et al., 2012].

On the other hand, the ephrin-B reverse signaling activated by postsynaptic EphB2 has an important implication in the development of retinotectal synapses [Lim et al., 2008].

Moreover, recent evidence has shown that EphA receptors are also implicated in the development and remodeling of synaptic structures through a form of neuroglial crosstalk. EphA4, expressed in dendritic spines of pyramidal neurons in the hippocampus, has a critical role in the regulation of postsynaptic morphology by interacting with ephrin-A3 ligand localized on the astrocytes [Murai et al., 2003].

Eph receptors and ephrins play a role not only in neurons and glial cells, but also in endothelial cells: EphB4, abundantly expresses in endothelial cells of embryonic veins, interacts with ephrin-B2, expressed in endothelial cells of arteries, contributing to vessel formation in the brain as well as the rest of the body.

1.4.2 Central Nervous System (CNS)

The Eph-ephrin system is not only expressed in the CNS of the embryo but it is also present in adult brain, particularly in areas where neuronal circuits are continuously remodeled in response to environmental changes [Yamaguchi et al., 2004]. For instance, Eph signaling controls neuronal plasticity in the hippocampus by regulating synapse number and size, and consequently taking part in learning and memory processes [Fig. 2].

Different Eph receptors and ligands are involved, depending on the brain region and sometimes even depending on the synapses in the same neuron [Dalva et al., 2007]. All the EphB receptors and EphA4 are the highly expressed in the hippocampus and can interact with a number of ephrins [Tremblay et al., 2007].

Alteration in the Eph-ephrin system could lead to mental retardation and dendritic spine abnormalities. Moreover, it has been found that downregulation of EphB2, due to the soluble β amiloyd protein, could contribute to neuronal degeneration and memory loss in the Alzheimer's disease [Lacor et al., 2007].

On the other hand, upregulation of the Eph-ephrin system has been observed in injured areas of the nervous system, suggesting a role in nerve regeneration [Du et al., 2007]. The Eph-ephrin system is not only involved in the re-arrangement of new connections and the axon re-growth (through repulsive signaling), but it is also expressed and activated in the inflammatory cells and fibroblasts around the injury [Wu et.al, 2007; Pasquale, 2008].

EphA4 has been found to be overexpressed in injured corticolspinal axons and astrocytes [Du et al., 2007; Fabes et al., 2007] after a damage to the spinal cord. Recent in vivo experiments performed in rats suggest the involvement of EphA4 forward signaling in axon retraction after injury and the subsequent regeneration and repair of the lesion. Eph receptor activation, mediated by ephrin-B2 expressed in astrocytes and ephrin-B3 expressed in myelin, also contribute to the formation of the glial scar [Pasquale, 2008].

A recent report shows a correlation between the EphA4 receptor and amyotrophic lateral sclerosis (ALS), a neurodegenerative disease affecting motor neurons. Low EphA4 activity seems to be protective and attenuates the degeneration of neurons. First, EphA4 knockdown reduces mutant SOD1-induced motor axonopathy. In addition, the receptor is physiologically involved in the regulation of the glial glutamate

transporter GLT-1, which is downregulated in ALS. Lastly, loss of EphA4 reduces the axonopathy due to mutations of TAR DNA-binding protein 43 (TDP-43) and due to the neuronal degeneration of motor neuron 1, two components involved in ALS pathogenesis, increasing the survival [Van Hoecke et al., 2012].

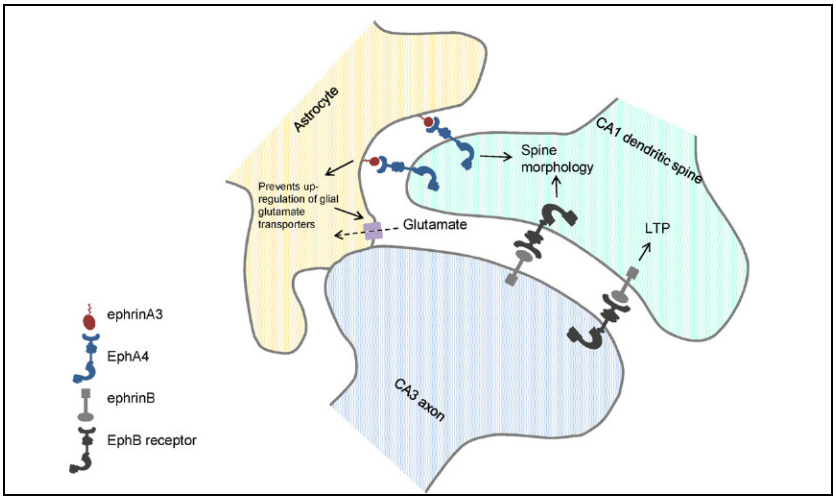


Figure 2: Eph-ephrin system in the CNS. Eph-ephrin signaling controls spine morphology and long term potentiation (LTP) in hippocampus. Post synaptic EphA4 interacts with astrocytic ephrin-A3. EphB receptors and ephrin-B ligands are also important for the regulation of the spine morphology [Attwood et al., 2012].

1.4.3 Platelet aggregation

Eph receptors and ephrins are expressed in human platelets and are involved in the maintenance of haemostatic plug stability and growth [Fig. 3]. In particular EphA4 and EphB1 are highly expressed on human platelets and play a key role by interacting with ephrin-B1.

During the platelet aggregation process, platelets increase the localization of $\alpha_{IIb}\beta_3$ integrins and Eph receptors at contact sites. The clustering of EphA4 or ephrin-B1 contributes to the activation of $\alpha_{IIb}\beta_3$ integrins by phosphorylating of the integrin cytoplasmic domain. In this way, the Eph-ephrin system promotes platelet adhesion/aggregation and it also contributes to clot retraction thus increasing the thrombus stability [Prevost et al., 2005; Brass et al., 2005].

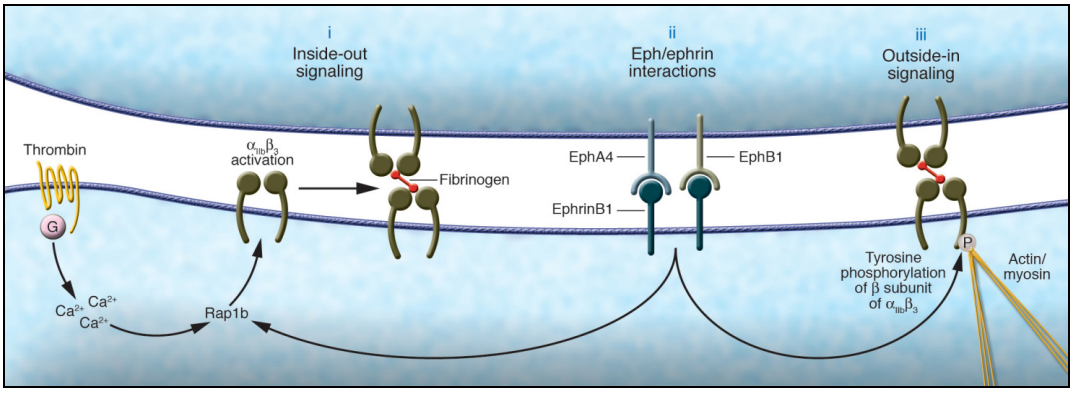


Figure 3: Eph-ephrin system in platelet aggregation. The interaction between EphA4 and EphB1 with ephrin-B1 in platelets is able to activate integrins contributing to cell-cell adhesion and aggregation of the platelets as well as to stabilize the thrombus [Brass et al., 2005].

1.4.4 Bone homeostasis

Multiple findings over the years have highlighted the role of the Eph-ephrin system in bone remodeling and maintenance [Zhao et al., 2006; Pasquale, 2008]. Bones are continuously remolded throughout life by the constant activity of osteoblasts and osteoclasts which are able to produce or degrade bone, and a proper balance has to be maintained in order to avoid diseases like osteoarthritis or cancer. Eph receptor/ephrin knockout mice show bones abnormalities and defects in skeletal development [Davy et al., 2006; Pasquale et al., 2005].

Cell-cell interaction is essential in the bone remodeling process, so that the Eph-ephrin system plays a key role in bones. Ephrin-B2 is highly expressed in osteoclasts and the interaction with EphB4 expressed by the osteoblasts suppresses osteoclastic bone resorption and increases osteoblatic bone growth. Eph receptors and ephrins of the A class are also involved in this process. Ephrin-A2 reverse signaling in osteoclasts increases osteoclastogenesis while EphA2 signaling inhibits bone formation and remineralization [Fig. 4] [Matsuo et al., 2012].

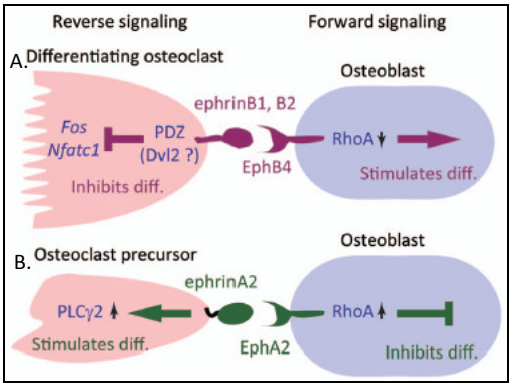


Figure 4: Eph-ephrin system in bone homeostasis. A. The interaction between osteoblastic EphB4 and osteoclastic ephrin-B2 regulates bone resorption and bone growth by regulating the differentiation of the two cell types. **B.** EphA2/ephrin-A2 interaction controls osteoclastogenesis [Matsuo et al., 2012].

1.4.5 Intestinal homeostasis

Eph receptors and ephrin ligands are commonly expressed in adult stem cell niches. They have been detected in hair follicle bulge, the mammary gland, neurogenic areas of the adult brain (subventricular zone and dentate gyrus of the hippocampus), and the intestine [Nikolova et al., 1998; Genander et al., 2010; Qiu et al., 2008].

The homeostasis of the intestinal epithelium is guaranteed throughout life by the balance of cell proliferation, differentiation and apoptosis and stem cells localized in the intestinal crypts can constantly proliferate [Barker et al., 2007].

EphB signaling is particularly relevant being able to control proliferation and migration processes in adult intestinal stem cell niches. Stem cells are found at the bottom of small intestine and colon crypts, where they divide frequently to generate progenitor cells, which migrate toward the top of the crypt and differentiate [Fig. 5] [Genander et al., 2010]. Interestingly, intestinal stem cells show high expression of EphB receptors (mostly EphB2 and EphB3), which is gradually lost as they become differentiated. In contrast, an increase of ephrin-B ligand expression has been shown in differentiated cells, generating an EphB/ephrin-B countergradient along the crypt axis [Pasquale 2008].

Proliferation of stem cells in intestinal niches is regulated by EphB2 in a kinase dependent pathway via Abl and cyclin D1, while migration is mediated by the PI3-kinase activity [Genander et al., 2010].

The EphA/ephrin-A system is less highly expressed in colonic crypts and probably it has a role in the regulation of the intestinal epithelial barrier [Kosinski et al., 2007; Rosenberg et al., 1997].

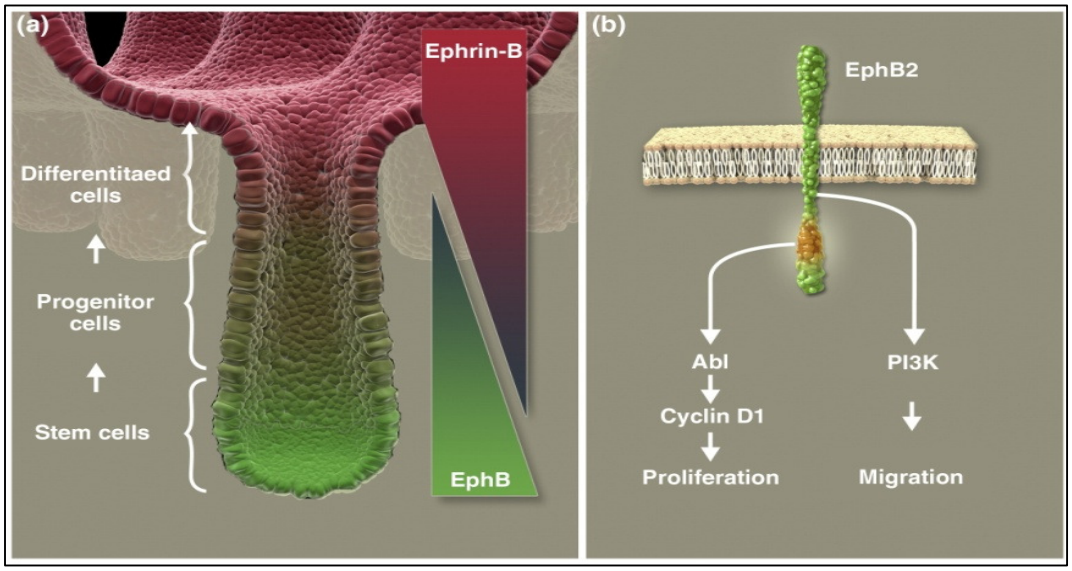


Figure 5: Eph-ephrin system in the intestine. **a.** The countergradient between EphB in the stem cells and ephrin-B in differentiated cells leads to the correct migration and positioning of intestinal cells. **b.** Cell proliferation and migration involve two different, signal cascades generated by EphB2 stimulation [Genander et al., 2010].

1.4.6 Immune system

EphA4 knockout mice showed a high decrease in T cell numbers due to thymic cortex abnormalities during development [Muñoz et al., 2007]. This suggests an important role of Eph receptors and ephrin ligands in immune system where cell-cell contact is important: they are expressed not only in lymphoid organs and lymphocytes but also in thymocytes, leading to their development into mature T cells and the subsequent differentiation into peripherical effector cells [Wo et al., 2005].

The EphB system is critical in immune regulation, in particular EphB2, EphB3 and EphB6 are able to regulate T cell responses promoting proliferation, interferon γ production and cytotoxic activity [Pasquale, 2008].

Recent evidence also supports a role of the Eph-ephrin system in B lymphocytes, although the specific effects are still unclear. Especially, ephrin-A4 found in activated B cells in the tonsils seems to interact with EphA2 expressed by tonsil cells that are localized in crypts and have a dendritic phenotype [Aasheim et al., 2000].

1.4.7 Inflammation

Cell adhesion plays an important role during inflammation: at the beginning of an inflammatory process there is a decrease in endothelial cell adhesion in blood vessels, followed by the adhesion of circulating leukocytes to the vascular endothelium. At early stages, EphA2 receptor and ephrin-B2 are overexpressed in epithelial and endothelial cells suggesting a role of the Eph-ephrin system in permeability regulation of the blood vessels at the inflamed site [Ivanov et al., 2006].

Furthermore, Eph-ephrin interaction promotes the inflammatory extravasation of leukocytes. In the later stages of LPS-induced systemic inflammation in rats, there is a dramatic downregulation of some Eph receptors (EphA1, EphA3, EphB3, EphB4) commonly expressed by endothelial cells and leukocytes. This decrease supports leukocyte adhesion and transmigration in tissues, probably regulating integrins and MAPK pathway [Ivanov et al., 2006; Pasquale, 2005; Poliakov et al., 2004].

A recent study conducted by Funk and colleagues, pointed out a correlation between EphA2 activation and atherosclerosis. Indeed, the human atherosclerotic plaque shows a high expression of EphA2 and ephrin-A1 ligand in endothelial cells and leukocytes, due to the induction of atherogenic mediators. Moreover, when EphA2 is stimulated by recombinant ephrin-A1 in endothelial cells, an activation of proinflammatory and prothrombotic gene expression as well as increased monocytes adhesion and integrin expression have been observed [Funk et al., 2012].

1.4.8 Glucose balance

Recently it has been proposed that the Eph-ephrin system plays a role in the regulation of glucose homeostasis. Pancreatic β cells use EphA/ephrin-A bidirectional signaling to modulate insulin secretion [Kostantinova et al., 2007]. In particular ephrin-A, mainly expressed on cells surface, increases insulin secretion through reverse signaling, while EphA receptors, which are also expressed in the intracellular secretory granules, are able to inhibit insulin secretion by means of forward signaling.

This process acts as a sort of feedback loop that lead to a balance between glucose levels, insulin secretion and phosphorylation/dephosphorylation of Eph receptors and ephrin ligands. When glucose is low EphA forward signaling prevails decreasing insulin

secretion. When glucose levels are high, EphA receptors are dephosphorylated, leading to downregulation of forward signaling without inhibition of ephrin-A reverse signaling which becomes predominant potentiating insulin secretion [Fig. 6] [Pasquale, 2008]. EphA5 and its cognate ligand ephrin-A5 which are coexpressed in β cells are particularly important for this process [Merlos-Suarez et al., 2008].

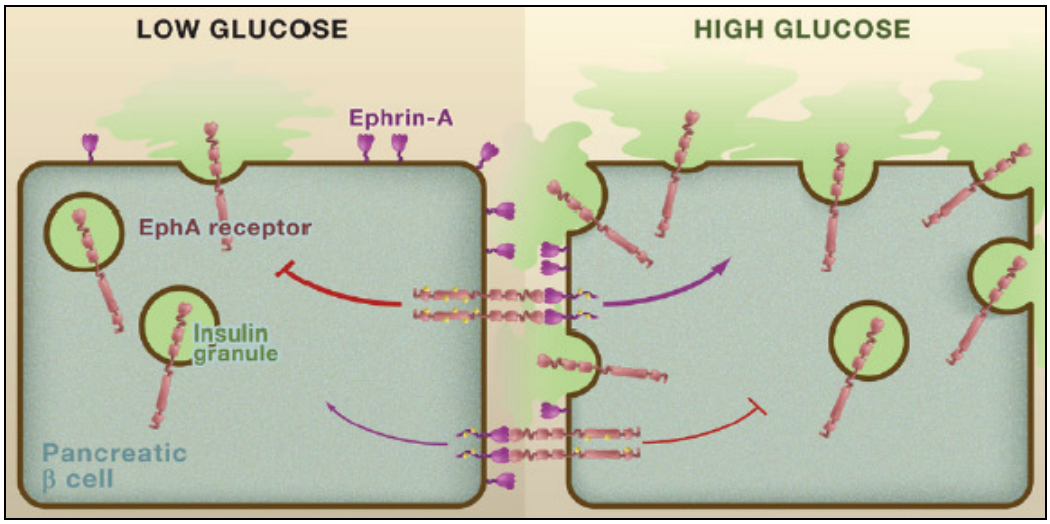


Figure 6: Eph-ephrin system in the regulation of glucose balance. EphA forward signaling prevails when glucose levels are low, leading to the inhibition in insulin secretion. Conversely, when glucose levels are high ephrin-A reverse signaling induces the release of the insulin from the granules [Pasquale, 2008]

1.4.9 Angiogenesis

The role played by the Eph-ephrin system in vascular development has been well characterized in the past years. During embryogenesis, the early vascular network (primary capillary plexus), derived from mesodermal angioblasts, forms the first primitive blood vessels, starting the initial process called vasculogenesis. In these initial stages, different RTKs are involved, in particular the VEGFR pathway. Subsequently, the primary plexus is remodeled generating a complex network of small and large

vessels through sprouting from the existing primitive vasculature: this second stage is called angiogenesis [Risau, 1997; Adams et al., 2000] .

Experiments in mutant mice showed the critical role played by Eph receptors and ephrin ligands in the angionenetic and lymphangiogenetic processes during development [Wang et al., 1998; Makinenet al., 2005]. Moreover, *in vivo* models demonstrated their role in pathological angiogenesis in the adult [Brantley et al., 2002]. In particular, the role of the Eph-ephrin system in angiogenesis was discovered through *in vivo* rat corneal angiogenesis experiments, which demonstrated the importance of ephrin-A1 as a trigger of angiogenesis in cornea while *in vitro* experiments demonstrated ephrin-A1-dependent chemotaxis of endothelial cells expressing EphA2 [Pandey et al., 1995].

Members of the Eph receptor and ephrin-B class seem to be the most involved in the early stages of development. Ephrin-B2 has been identified as a marker of arterial endothelial cells, while EphB4 is expressed by venous endothelial cells [Wang et al., 1998; Adams et al., 2000] and interactions between the two proteins is crucial for the remodeling and separation of the vasculature at the arterial-venous boundary [Fig. 7]. EphB4 or ephrin-B2 knock out mice die during embryonic stages due to severe cardiovascular defects [Wang et al., 1998; Gerety et al., 1999].

Ephrin-B1 is also expressed by endothelial cells of both arterial and venous embryonic vessels, while ephrin-B3 is mainly expressed in the heart, where probably it plays a role in cardiovascular development by interacting with both EphB4 and EphB3 [Adams et al., 2000; Brückner et al., 1999]. EphB3 exists in both venous and arterial blood vessels and is able to interact with the three B ephrins, making the dynamics of interaction even more complicated.

The interplay between endothelial cells and the mesenchimal cells of the surrounding tissues expressing the EphB2 receptor is also remarkable. The interaction of EphB2 with ephrin-B ligands at the vessels boundary regulates the organization of the entire vessel [Adams et al., 1999].

The EphA/ephrin-A system also takes part in the angiogenesis process. EphA receptors mediate angiogenic responses through the activation of Vav exchange factors and the Rho GTPase Rac1 as well as the PI3K pathway. When EphA2 is activated by ephrin-A1 stimulation, Vav proteins are recruited to the receptor and subsequently activate the Rac1 GTPase. This process leads to cytoskeletal modifications therefore regulating angiogenic response [Hunter et al., 2006]. It has been also found that when EphA receptors are stimulated by ephrin-A1 ligand, they are

able to regulate the vascular permeability in the lung [Larson et al., 2008]. The EphA2 is required for VEGF-induced endothelial cell migration leading to the formation of capillary tube structures [Chen et al., 2006]. The EphA2 receptor is not expressed in the embryonic vasculature or the adult quiescent vasculature, but the interaction with ephrin-A1 expressed in tumor endothelial cells activates endothelial EphA2 [Pasquale 2008].

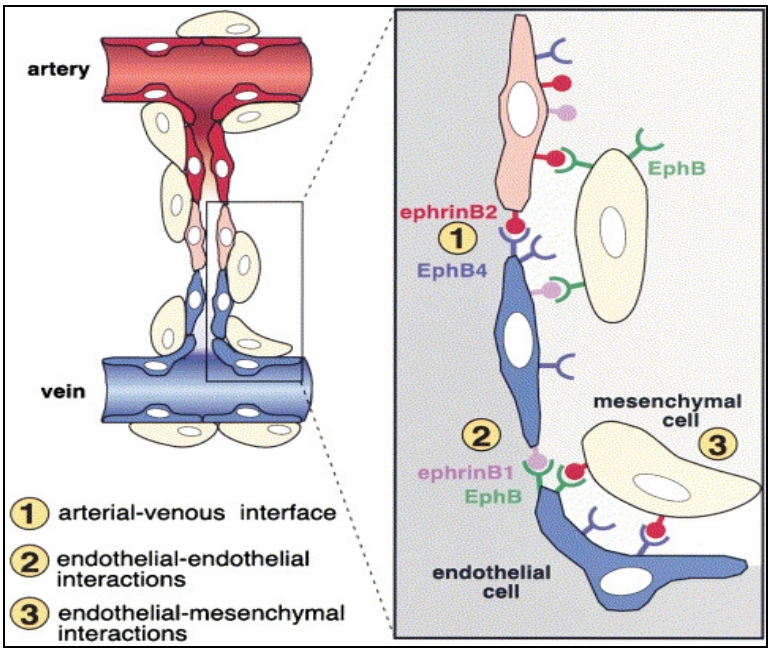


Figure 7: Eph-ephrin system in angiogenesis. The interaction between EphB4 expressed by venous endhothelial cells and ephrin-B2 in the arterial endothelial cells outlines the vasculature at the arterial-venous boundary, while the interaction between ephrin-B1 of endothelial cells and EphB receptors expressed by neighbor endothelial and mesenchimal cells leads to vascular remodelling [Adams et al., 2000].

1.5 Eph-ephrin system in cancer progression

The role of the Eph-ephrin system in tumorigenesis is still controversial. Opposite effects have been found, depending on tissue, cellular context and pathway involved. Eph receptors and ephrin ligands are often overexpressed in many different kinds of tumors and their upregulation is associated with tumor growth and survival as well as invasion and metastasis [Noren et al., 2007; Surawska et al., 2004; Brantley-Sieders et al., 2004]. On the other hand, a tumor-suppressor activity has been reported for Eph signaling in some tumors [Noberini et al., 2009; Vaught et al., 2009].

Despite this, high levels of several Eph receptors in cancer cells are correlated with a poor prognosis, high mortality and increased metastasis, identifying this system as an emerging target for cancer therapy [Pasquale, 2008; Tandon et al., 2011; Kandouz, 2012]. In particular, the EphA2 and EphB4 receptors are the most extensively studied in the cancer field and they are often overexpressed in tumors [Walker-Daniels et al., 2003; Kinch et al., 2003; Wykosky et al., 2008; Brantley-Sieders et al., 2011].

A number of reports showed high levels of EphA2 in solid tumors, such as breast cancer, liver cancer, prostate cancer, ovarian cancer, melanoma and glioblastoma, in some cases correlating with an aggressive phenotype [Surawska et al., 2004]. EphA2 overexpression is sufficient to induce tumorigenesis in mammary epithelial cells when EphA2 fails to interact with its ligand [Zelinsky et al., 2001].

EphA2–ephrin-A1 signaling controls many pathways that are involved in the oncogenic process, for instance the PI3K pathway, Src family kinases, Rho and Rac GTPases, the MAPK pathway and integrins. Moreover, there are correlations with other oncogenic receptors like EGFR, which regulates cell proliferation. Cell-cell contact is crucial for the activation of the bidirectional signaling, but some reports showed that the soluble and monomeric ephrin-A1 released by tumor cells can also activate the receptor [Wykosky et al., 2008]. After the interaction, EphA2 receptor dimerizes and becomes phosphorylated in tyrosine residues of the kinase domain starting the signals. The complexity of EphA2–ephrin-A1 interaction is due to the multiple, and in some cases context-dependent, signaling cascades that can be activated by this receptor [Tandon et al., 2011]. For instance, the activation of EphA2 overexpressed in the PC3 cell line is associated to the activation of SHP2 (SH2 domain-containing tyrosine phosphatase), which is able to dephosphorylate FAK (Focal Adhesion Kinase) leading to decreased cell adhesion [Miao et al., 2000]. On the other hand, other studies showed an inhibition of the cell proliferation in prostatic epithelial cells and endothelial cells treated with ephrin-A1, due to inhibition of the Ras/MAPK pathway [Miao et al., 2001].

Furthermore, EphA2–ephrin-A1 interaction is crucial for pathological angiogenesis, which is important for tumor growth and metastasis. EphA2 is poorly expressed in the embryonic endothelial cells but it is overexpressed in tumor endothelial cells, suggesting an important role in the development of the tumor microenvironment [Brantley-Sieders et al., 2004].

1.5.1 Colorectal cancer

Alterations in the physiological self-renewal of intestinal cells can generate malignant transformation. Many reports highlighted an overexpression of Eph-ephrin proteins in colorectal cancers, including EphA2 [Ogawa et al., 2000], EphA1 [Herath et al., 2009] and EphB4 [Stephenson et al., 2001; Herath et al., 2010]. The Eph receptors and ephrins are especially localized in the epithelium on the luminal surface, a cell layer that could potentially give rise to metastasis [Liu et al., 2002; Stephenson et al., 2001] and EphA2 in particular is overexpressed in the early stages of cancer [Herath et al., 2010; Kataoka et al., 2004].

While EphB2 is expressed in normal colonic cells and its expression is correlated with a prolonged survival, EphB4 expression is deficient in the normal colon; however, EphB4 has been detected in tumor cells and its expression level is associated with higher tumor stage and grade [Kumar et al., 2009]. EphA1 is considered a potential prognostic marker in colorectal cancer [Herath et al., 2009] and EphA2 overexpression is often associated with metastasis. Indeed, recent data correlated high levels of EphA2 in metastatic colorectal cancer with a loss of E-cadherin expression and liver metastasis [Saito et al., 2004], showing an inverse relationship that could be critical for the metastatic process.

1.5.2 Breast cancer

EphA2 and EphB4 are the most studied Eph receptors in breast cancer. During mammary development the receptors are mostly distributed in the myoepithelial cells that are localized around the ducts and alveoli [Brantley-Sieders et al., 2004]. In cancer cell lines, these receptors are phosphorylated at low levels suggesting low ephrin-induced activation that may be due to the low levels of the ligands [Pasquale, 2008].

Many reports showed EphA2 and EphB4 overexpression in human mammary epithelial cell lines, suggesting roles in tumor formation, but opposite effects on carcinogenesis have also been reported [Noren et al.,2007]. Indeed, EphB4 forward

signaling seems to inhibit breast cancer cells tumorigenicity, cell growth and migration. However, EphB4 has also been associated with cancer progression, for example by promoting angiogenesis through stimulation of ephrin-B2 reverse signaling [Noren et al.,2007]. In addition, EphB4 knockout mice show a decrease in tumor cells migration, invasion and proliferation, not only in breast cancer but also in other cancer types [Kumar et al., 2006; Noren et al., 2007].

Perhaps, the explanation of these conflicting data has to be found in the oncogenic signaling pathways responsible for transformation and in context dependent activities of the EphB4 receptor in breast tumors, but further studies are necessary for a complete understanding of Eph-ephrin activities in breast cancer.

1.5.3 Prostate cancer

EphA2 overexpression has been observed in prostate cancer cells, especially in metastatic prostate cancer cells where it has been detected at 10-100 times higher levels compared to non-invasive prostate epithelial cells [Walker-Daniels et al., 1999].

While EphB3 and EphB4 overexpression is involved in the loss of contact inhibition of locomotion and invasiveness in prostate cancer cells [Astin et al., 2010], EphB2 seems to have a role as a tumor suppressor in the prostate because EphB2 mutations that decrease receptor expression lead to cancer progression and metastasis [Huusko et al., 2004].

2. Targeting the Eph-ephrin system: emerging strategies

The role played by the Eph-ephrin system in cancerogenesis and other pathological conditions, offers the opportunity to target this system for therapeutic applications. An elevated Eph receptor expression in cancer supports the possibility of inhibiting Eph-ephrin signaling in tumor cells and in the vasculature surrounding the tumor, in order to prevent angiogenesis. The EphA2 receptor is particularly interesting in this context because it is able to regulate various critical aspects of oncogenesis (including cell adhesion, survival, transformation and migration) and it is often associated with metastasis and poor prognosis [Pasquale, 2010; Brantley-Sieders, 2012; Beauchamp et al., 2012].

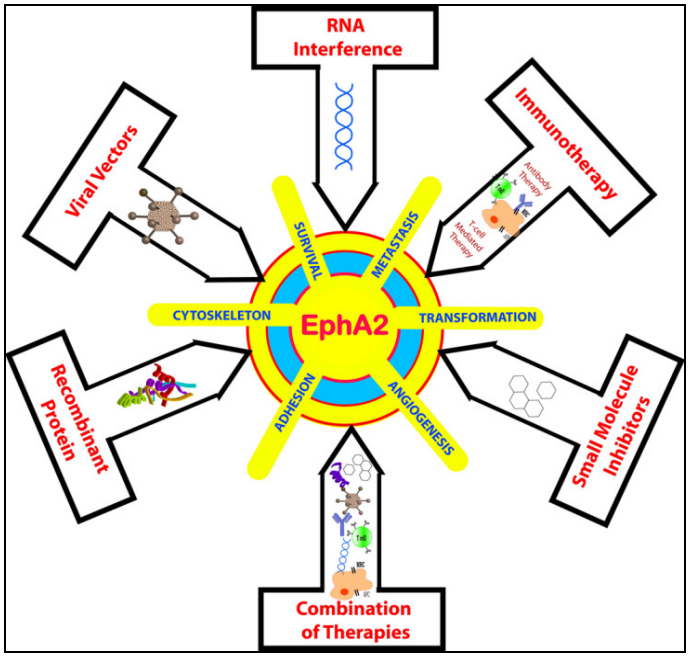


Figure 8: Targeting the Eph-ephrin system. Eph receptors and ligands are involved in a variety of pathological processes in many tissues. Different strategies have been suggested during the past years to manipulate this system in order to treat many diseases such as cancer [Tandon et al., 2011].

A number of therapeutic strategies have been proposed during the years to target the Eph receptors [Fig. 8]: monoclonal antibodies [Carles-Kinch et al., 2002],

immunoconjugates [Lee et al., 2009; Lee et al., 2010], immunotherapy [Kawabe et al., 2009], recombinant proteins [Wykosky et al., 2008], viral vectors [Sharma et al., 2008], RNA interference [Kikawa et al., 2002] and nanoparticles [Couvreur et al., 2006].

The development of small molecules represents an attractive alternative. Two main approaches have been followed to inhibit Eph-ephrin signaling with small molecules: inhibition of forward signaling with selective kinase inhibitors and disruption of Eph-ephrin interaction [Fig. 9].

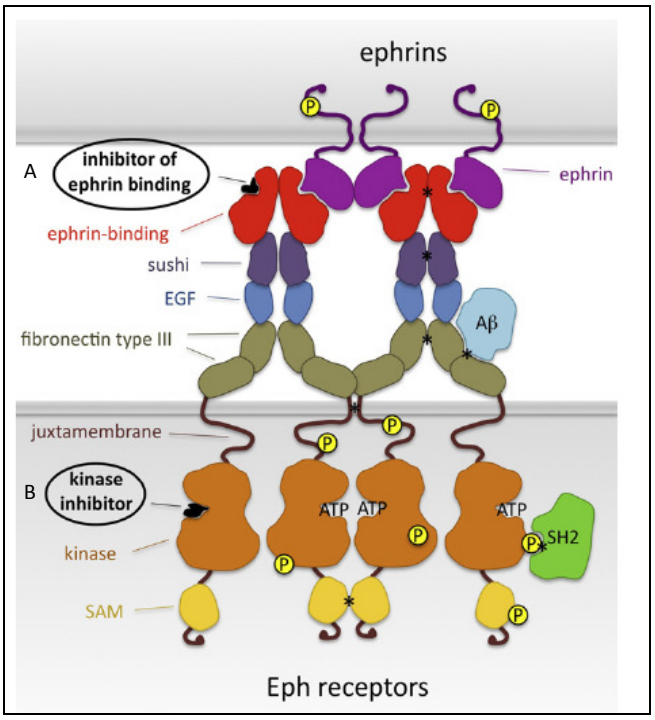


Figure 9: Strategies to target Eph receptors with small molecules. **A**. Small molecules can interact with Eph receptors in the extracellular receptor ligand binding domain (LBD) thus blocking the ephrin binding in the site; **B**. Kinase inhibitors can interact with the kinase domain situated in the intracellular site to block Eph receptor kinase activity [Noberini et al., 2011].

2.1 Kinase inhibitors

Many kinase inhibitors can potently inhibit the Eph receptor kinase domain, but these compounds lack selectivity and they usually interact with other classes of kinases [Huang et al., 2007].

Dasatinib is one of the Eph kinase inhibitors able to interfere with the ATP-binding pocket of the receptor. It was developed originally to target Bcr-Abl and Src family

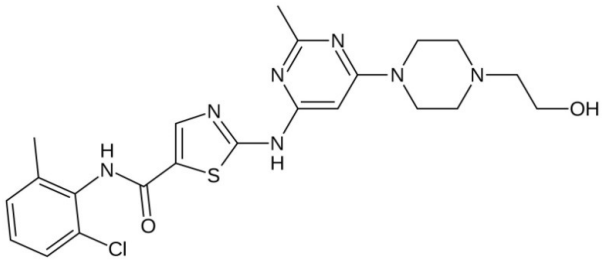
kinases, but it showed a significative activity also on the EphA2 kinase. Dasatinib is not selective for the Eph-ephrin system, but it is a multi-target kinase inhibitor able to block EphA2 phosphorylation in a dose-dependent manner when stimulated with ephrin-A1 ligand. It is also able to inhibit the ligand-induced binding of EphA2 to the ubiquitin ligase Cbl and EphA2 internalization/degradation. Moreover dasatinib was tested in BxPC-3 xenografts showing that a treatment with 50 mg/Kg in a single dose can inhibit EphA2 phosphorylation after 2 and 4 hours [Chang et al., 2008].

Nilotinib was identified as an EphB2 protein kinase inhibitor through a proteomic and cellular screening approach, and is more selective for the Eph-ephrin system than dasatinib. Starting from its structure, Choi and colleagues developed a combinatorial library of molecules able to interact with the Eph receptors on the kinase domain [Choi et al., 2009].

By screening a kinase inhibitor library, 2,4-bis-anilinopyrimidine derivatives have been identified as active compounds that inhibit the EphB4 kinase domain at nanomolar concentrations, although they also have a good activity on the Src kinase family [Bardelle et al., 2008; Bardelle et al., 2010; Barlaam et al., 2011]. EphB4 is also a target for new inhibitors identified through a high-throughput in silico docking using the EphB4 kinase domain as a scaffold [Lafleur et al., 2009].

A low kinase selectivity of the compounds could be useful in some cases, because in many tumors a large number of RTKs are alterated and moreover a multi-target therapy could prevent development of resistance [Zhang et al., 2009].

However, studies carried out on the ATP binding pocket of human kinases supported the possibility to design selective inhibitors for Eph receptors [Huang et al., 2007]: for instance, cysteine residues close to the P loop of many Eph receptors kinase are absent in many other kinases [Zhang et al., 2009].



Dasatinib

2.2 Molecules targeting on the LBD

2.2.1 Peptides

Through a phage display screening, a serie of 12 aminoacid-long peptides have been identified that are able to bind the extracellular ligand binding domain of the Eph receptors showing micromolar affinity [Koolpe et al., 2002; Koolpe et al., 2005; Murai et al., 2003]. This was the first approach for the identification of molecules capable to interact with Eph receptors directly in the ligand binding pocket. Three of these peptides (KYL (KYLPYWPVLSSL), APY (APYCVYRGSWSC) and VTM (VTMMEAINLAFPG)) showed high affinity for the EphA4 receptor and they can inhibit ephrin-A binding and EphA4 activation. KYL is the most potent, with an IC_{50} value of 1 μM. In addition, in vitro experiments demonstrated the ability of the peptides to disrupt the physiological function of endogenous EphA4 *in situ* [Murai et al., 2003].

Two of the peptides identified with the phage display approach were selective for the EphA2 receptor: YSA (YSAYPDSVPMMS) and SWL (SWLAYPGAVSYR). In the Biacore analysis these peptides showed K_D values of 186 nM for YSA and 678 nM for SWL and they were both able to induce EphA2 phosphorylation in cellular assays, leading to receptor endocytosis. The ability of YSA and SWL peptides to specifically target EphA2 has been exploited for the delivery of anticancer drugs to tumor cells, by taking advantage of the high expression of the receptor in cancers and tumor blood vessels as well as its low levels in normal adult cells [Blackburn et al., 2009; Dickerson et al., 2010].

Other peptides able to interact with EphB receptors have been identified: SNEW (SNEWIQPRLPQH), which binds to the EphB2 receptor and TNYL (TNYLFSPNGPIA), which binds to the EphB4 receptor, both with micromolar affinity [Koolpe et al., 2005]. Modification of TNYL to TNYL-RAW by addition of the C-terminal RAW sequence was shown to improve potency to nanomolar affinity [Chrencik et al., 2007].

2.2.2 Lithocholic acid (LCA)

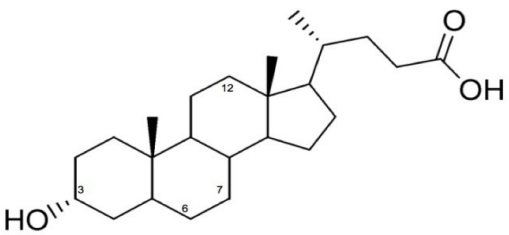
The use of peptides for therapy could be limited due to their poor pharmacokinetic profile, low stability *in vivo* and poor tumor penetration, even though they can be easily produced biosynthetically and they could have less toxicity than chemical compounds [Lien et al., 2003].

On the other hand, many groups have aimed at identifying small molecules able to interact with the ephrin-binding pocket of Eph receptors. This has turned out to be difficult owing to the large size of the Eph-ephrin interacting surface.

Lithocholic acid (LCA) was identified by our lab through an ELISA-based binding assay that was used to screen hundreds of compounds belonging to a in house-chemical library. LCA is a secondary bile acid, necessary for lipid solubilization and absorption in the intestine during digestion. Among all the bile acids tested, only LCA was able to dose-dependently disrupt the interaction between EphA2 receptor and ephrin-A1 ligand showing a Ki value of 49 µM. The compound is a pan Eph inhbitor being a promiscuous ligand of EphA and EphB receptors subfamilies.

LCA was found to be a reversible, competitive antagonist of Eph-ephrin system: in cellular assays performed on PC3 cells, T47D cells and HT29 cells it was able to dose-dependently inhibit the phosphorylation of Eph receptors, when treated with the physiological ligand, at not-cytotoxic concentrations. The compound antagonized Eph-kinase phosphorylation by inhibiting protein-protein interactions without affecting the kinase domain, moreover the inhibitory effect was specific for Eph receptors since LCA resulted completely inactive when tested for inhibition of phosphorylation of other kinases (EGFR, VEGFR, IRKβ or IGFR1β).

In functional *in vitro* assays, LCA inhibited PC3 cells adhesion to coated-ephrin-A1 and furthermore, it was able to inhibit PC3 cells retraction and rounding due to EphA2 stimulation with ephrin-A1 in a dose dependent manner [Giorgio et al., 2011].



Lithocholic acid

2.2.3 Salicylates

Recent studies showed the role of the salicylic acid group in targeting Eph receptors [Noberini et al., 2008; Noberini et al., 2011]. In a first study, a high throughput screening identified two isomeric salicylic acid dimethylpyrrole derivatives that were able to selectively inhibit the binding of ephrin-A5 to the EphA2 and EphA4 receptors in ELISA assays, showing IC_{50} values of 10 μM. These compounds were active also in functional assays performed on cells, including inhibition of receptor phosphorylation and PC3 cells retraction and rounding [Noberini et al., 2008]. Regrettably, the pyrrolyl ring group could be problematic because of its tendency to undergo modifications, and these compounds may polymerize or become oxidized, forming secondary products responsible of the inhibitory activity and difficult to isolate [Baell et al., 2010; Sink et al., 2010].

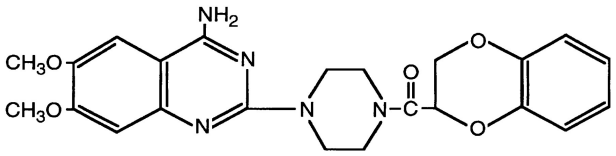
Accordingly, in order to identify compounds with similar characteristic but without the pyrrole and with increased potency, a second panel of salycilics was screened. In this library, a small molecule containing two salicylic acid-furanyl groups was discovered. This new compound was able to irreversible and selectively bind the EphA2 and EphA4 receptors interfering with ephrin-A5 binding and it was also able to inhibit the activation of Eph receptors in cellular assays [Noberini et al., 2011].

2.2.4 Doxazosin

A recent study reported the activity of doxazosin on the Eph-ephrin system [Petty et al., 2012]. Doxazosin is a well-known α1-adrenoreceptor antagonist, regularly used in the clinic for the treatment of hypertension and benign prostate hyperplasia (Cardura®). This molecule is a selective agonist for EphA2 and EphA4 receptors through a mechanism of action that is independent of α1-adrenoreceptor inhibition.

In binding assays performed through SPR analysis doxazosin showed a Kd value of 47.5 μM, while in cellular assays the compound was able to activate the EphA2 receptor at concentrations higher than 25 μM, increasing receptor internalization and inhibiting the migration of various tumor cell lines. *In vivo* experiments performed on

prostate cancer xenografts in mice treated daily with 50 mg/kg of doxazosin for 10 days showed reduced metastasis and increased survival.



Doxazosin

2.2.5 Polyphenols

In our ELISA screening, beside LCA we also identified a number of polyphenols rich-plant extracts able to inhibit EphA2–ephrin-A1 binding with IC_{50} values in the range of 0.83-24 µg/ml. In functional assays performed on PC3 cells, the active extracts specifically blocked EphA2 phosphorylation induced by ephrin-A1 stimulation in a dose-dependent manner and at not-cytotoxic concentrations [Mohamed et al., 2011].

Previous studies reported the antagonistic properties of epigallocatechin-3-gallate (EGCG), a green tea derivative, on EphA2 and its inhibitory effect on endothelial cell migration and capillary-like tube formation [Tang et al., 2007]. Recent reports have revealed that theaflavin monogallates from black tea and epigallocatechin-3,5-digallate from green tea are also able to modulate the Eph-ephrin system [Noberini et al., 2012].

AIM

The Eph-ephrin system is involved in a variety of physiological and pathological processes. Therefore, identification of molecules able to modulate this system represents a novel and attractive approach for the treatment of some diseases, including cancer [Surawska et al., 2004; Liu et al., 2007].

Indeed, EphA2 and EphB4 receptors are upregulated in several tumors. High levels of EphA2 are often correlated with tumor progression and dissemination [Ogawa et al., 2000; Walker-Daniels et al., 2003], in association with increased tumor angiogenesis [Brantley-Sieders et al., 2004]. Based on these findings, strategies to interfere with EphA2 signalling have been explored over the years. Development of small molecules able to inhibit Eph-ephrin binding by targeting the Eph LBD is one of the possible approaches.

The identification of LCA as a reversible, competitive antagonist of the Eph-ephrin system identified a new scaffold that could be useful for the design of compounds able to target the Eph-ephrin system with higher affinity and potency.

Following this strategy, new bile acids derivatives have been synthesized and examined for their ability to disrupt EphA2–ephrin-A1 binding and, analyzing this new series of molecules, a structure-activity relationship (SAR) has been obtained leading to the identification of compounds with improved binding affinity.

Moreover, the recent resolution of the crystal structure of the ligand binding domain of the EphA2 receptor in complex with ephrin-A1 [Himanen et al., 2009] and the analysis of the SAR of these new derivatives allowed modeling of LCA binding to the EphA2 receptor and extrapolation of the pharmacophoric group responsible for the activity.

The most active compounds identified have been pharmacologically characterized using functional assays performed on cells, with the goal to evaluate the antagonistic profile of these new derivatives. Moreover, cytotoxicity assays have been performed in order to determine the toxicity of the compounds on cells and to estimate their antiproliferative effect.

MATERIAL AND METHODS

1.Reagents

All culture media and supplements were purchased from Euroclone; recombinant proteins and antibodies were from R&D systems; cells were purchased from ECACC. Leupeptin, aprotinin,NP40, MTT, tween 20, BSA and salts were from Applichem; EDTA, sodium orthovanadate, 4',6-diamidino-2-phenylindole (DAPI) were from SIGMA. Human IgG fragment was from Millipore; Matrigel was purchased from BD Bioscience and rodhamine-phalloidin from Invitrogen.

2. Cell culture

PC3 human prostate adenocarcinoma cells were manteined in Ham F12 supplemented with 5% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution. HT29 human colon adenocarcinoma cells were grown in EMEM supplemented with 10% FBS, 1% non-essential aminoacids solution (NEAA), 1% sodium piruvate and 1% penicillin-streptomycin solution. T47D human breast cancer cells were grown in RPMI 1640 with 10% FBS and 1% penicillin-streptomycin solution. Human umbilical vein endothelial cells (HUVECs) were maintained in MEM 200 supplemented with 1% penicillin-streptomycin solution, 1% fungizone solution, 2% low serum growth supplement (LSGS) and 10% FBS. All the cell lines were grown in a humidified atmosphere of 95% air, 5% CO_2 at 37°C.

3. ELISA binding assay and Ki/IC_{50} determination

96 well ELISA high binding plates (Costar #2592) were incubated overnight at 4°C with 100 µl/well of 1 µg/ml EphA2-Fc (R&D 639-A2) diluted in sterile PBS (0.2 g/l KCl,

8.0 g/l NaCl, 0.2 g/lKH2PO4, 1.15 g/l Na2HPO4, pH 7.4), washed three times with washing buffer (PBS + 0.05% tween20, pH 7.5) and blocked with 300 µl/well of blocking solution (PBS + 0.5% BSA) for 1 hour at 37°C. Compounds were added to the wells at proper concentrations and incubated at 37°C for 1 hour. Biotinylated ephrin-A1-Fc (R&D BT602) was added at 30 ng/ml for 4 hours in displacement studies and 5 hours for saturation studies. Wells were washed three times and incubated with 100 µl/well streptavidin-HRP (Sigma S5512) solution (0.05 µg/ml in PBS supplemented with 0.5% BSA, pH 7.4) for 20 minutes at room temperature, then washed again for three times and incubated at room temperature with 0.1 mg/ml tetra-methylbenzidine (Sigma T2885) reconstituted in stable peroxide buffer (11.3 g/l citric acid, 9.7 g/l sodium phosphate, pH 5.0) and 0.02% H_2O_2 (30%, m/m, in water) added immediately before the use. The reaction was stopped with 3N HCl 100 µl/well and the absorbance at 450 nm was measured using an ELISA plate reader (Sunrise, TECAN, Switzerland).

The IC_{50} value was determined using one-site competition nonlinear regression and Kd values of the curves with or without antagonists were calculated using one-binding site non-linear regression analysis with Prism software (GraphPad Software Inc.). The Ki was obtained using Schild plot [Arunlakshana et al., 1959] where Log[DR-1] is a function of the negative Log_{10} of the inhibitor concentration. The Hill's coefficient was calculated using linear fitting to evaluate whether the inhibition was competitive or uncompetitive.

4. Cell lysates

Cells were grown in 12-well plates at the concentration of $3x10^5$cells/ml in complete medium until they reached 40% of confluence and then serum starved overnight. The day after cells were treated with compounds (or vehicle as a control) for 20 minutes, stimulated with the agonist for other 20 minutes, rinsed with cold PBS and solubilized in lysis buffer. The lysates were resuspended and rocked on ice for 30 minutes, then centrifuged at 14000xg for 5 minutes. The protein concentration of supernatant was measured with BCA protein assay kit (Thermo scientific), standardized to 200 µg/ml and transferred into a clean test tube ready to be used.

5. Evaluation of EphA2, EphB4, EGFR phosphorylation

EphA2-, EphB4- and EGFR-phosphorylation has been measured in cell lysates using DuoSet®IC Sandwich ELISA (RnD Systems, #DYC4056, #DYC4057 and #DYC1095, respectively) following manufacturer's protocol. A 96 well ELISA high binding plate (costar 2592) was incubated 100 µl/well overnight at room temperature with 4 µg/ml of the specific capture antibody diluted in sterile PBS. The day after wells were washed and blocked for 1 hour at room temperature. Then, lysates were added at room temperature for 2 hours and after another washing they are incubated with detection antibody at room temperature for other 2 hours. The amount of phosphorylation was revealed utilizing a standard HRP format with a colorimetric reaction read at 450 nm.

6. Kinase assay

Evaluation of the effects on human EphA2 kinase was performed by measuring the phosphorylation of substrate Ulight-TK peptide (50 nM) using the LANCE detection method [Olive , 2004]. Staurosporine was used as reference compound.

7. MTT assay

Cell viability was evaluated using the MTT colorimetric assay. Cells were plated in 96-well plates at a density of 10^5 cells/ml and the day after treated with compounds or 0.5% DMSO for 24, 48, or 72 hours. MTT was added at the final concentration of 1 mg/ml and incubated for 2 hours. The resulting formazan crystals were solubilized with DMSO and the absorbance was measured at 550 nm using an ELISA plate reader. The results were expressed as the ratio between absorbance of the cell treated with the compounds and untreated cells.

8. LDH assay

Cytotoxicity of the compounds was measured using CytoTox 96 Non-Radioactive Cytotoxicity Assay, following the manufacturer's protocol (Promega 1780). Cells were seeded in 96-well plates at a density of 10^5cells/ml, the day after they were treated with compounds or lysis buffer for 2 or 24 hours. The released LDH in culture supernatants was evaluated incubating the reactive solution for 30 minutes where the tetrazolium salt (INT) of the buffer is converted into a red formazan product. The colorimetric reaction was quantified by an ELISA plate reader (Sunrise, TECAN, Switzerland) reading at 492 nm. Results were expressed as the ratio between the absorbance of cells treated with compounds and cells treated with lysis buffer.

9. Retraction assay

PC3 cells were grown at the concentration of $4x10^4$ cells/well on 96-well culture plates (Greiner Bio One) and starved for 2 hours before the experiment. Cells were treated with compounds or DMSO for 20 minutes then stimulated with 0.5 µg/ml ephrin-A1 (or Fc) for additional 20 minutes. Subsequently, cells were fixed with formaldehyde 3.7% solution for 15 minutes, permeabilized with 0.5% Triton for 3 minutes, blocked 1 hour with 10% goat serum and stained with rodhamine-phalloidin and DAPI solution.

10. Tube formation assay

A 24 well-plate for tissue culture was coated with BD Matrigel 80µl/ well for 30 minutes at 37°C in order to form a thin layer of gel on the bottom of wells. Then HUVECs were treated with the compounds (or DMSO as control) and immediately seeded at the concentration of $3.2x10^5$ cells/well. After 15 hours they were fixed with formaldehyde 3.7% 15 minutes at room temperature, and then pictures were taken.

11. Surface Plasmon Resonance (SPR)

The SPR (Surface Plasmon Resonance) assay ws performed using the Biacore Technology [Giannetti et al., 2008]. Solubilized compounds were incubated on a surface pre-coated with proteins (EphA2-Fc, ephrin-A1/B1-Fc, Fc) and optical biosensors linked to the surface [Pini et al., 2007], revealing mass changing, detected variations when the molecule bound the surface. The signal was recorded through a sensogram and was measured in RU (Resonance Units). K_D values could be extrapolated from the sensogram, using a steady state-analysis performed by plotting the binding at equilibrium versus the ligand concentration and assuming that the K_D equals to the concentration which yields 50% of the maximum response [Nuñez et al., 2012].

12. Statistical analysis

Data are the means of at least three independent experiments ± standard errors.
One way ANOVA followed by Dunnet's post test was performed in phosphorylation assays, retraction assays and tube formation assays. Significativity is considered when $P< 0.05$.

RESULTS

1. Preliminary data: hit discovery

In order to properly understand the results of this study, an introduction showing some preliminary data related to the discovery of a hit compound, namely lithocholic acid (LCA), could be useful [Giorgio et al., 2011].

1.1 LCA is a competitive and reversible Eph receptor ligand

Through an ELISA-based binding screening, LCA was identified as a small molecule able to interfere with Eph-ephrin binding amongst two thousands compounds screened. Lithocholic acid was able to dose-dependently inhibit biotinylated ephrin-A1 binding to the immobilized EphA2 receptor, showing an IC_{50} value of 57 µM [Fig. 10 A]. Interestingly other bile acids tested (cholic acid (CA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA)) failed to affect EphA2–ephrin-A1 binding. Saturation studies were then performed and curves of EphA2–ephrin-A1 binding in the presence of increasing concentrations of LCA were plotted [Fig.10 B]. Non linear regression was used to extrapolate the value of the dissociation costant (K_D) or apparent K_D for each curve. These values were used to obtain the Schild plot [Fig.10 C], where Log[DR-1] was a function of -log10 [inhibitor] [Arunlakshana et al., 1959]. The interpolated regression line (r^2=0.9664) showed a slope value of 0.8618 suggesting a competitive

binding. We derived also the pKi value calculating the intersection of this interpolated line on the X axis, which was 4.31±0.03 and corresponded to a Ki of 49 µM.

Displacement studies were repeated by incubating 200 µM LCA for 1 hour and washing some of the wells before adding 50 ng/ml biotinylated ephrin-A1. Displacement of biotinylated ephrin-A1 was detected only in the unwashed wells suggesting the reversibility of binding to the EphA2 receptor [Fig.10 D].

LCA activity towards all the EphA and EphB kinases was also tested using biotinylated ephrin-A1 or ephrin-B1, respectively: the compound did not discriminate between the two receptors subclasses and bound promiscuously to both subclasses [Fig.11 A, Fig.11 B].

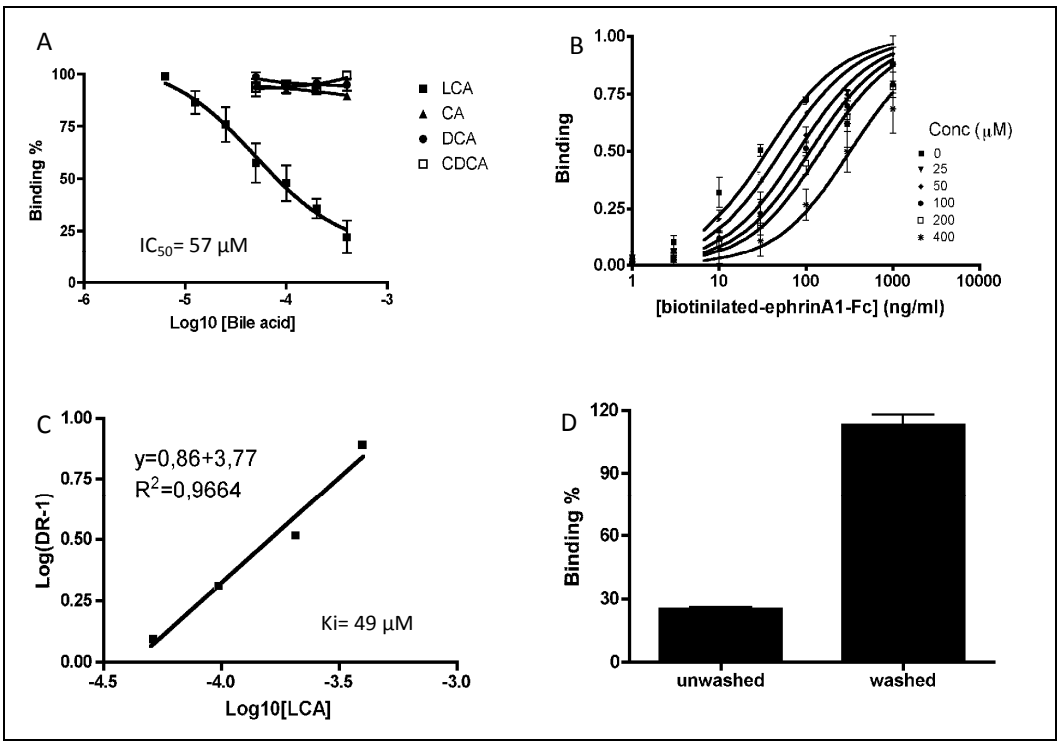
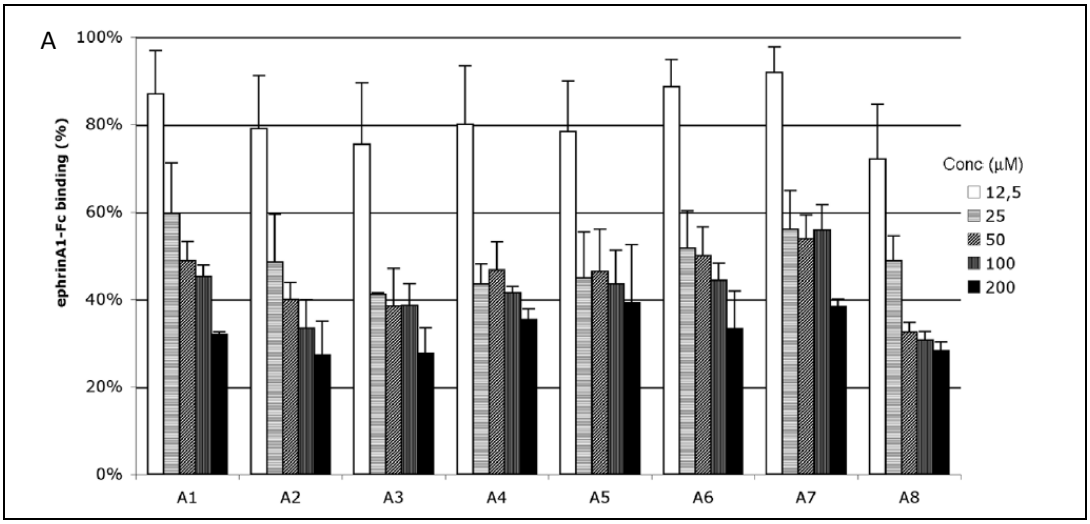


Figure 10: Lithocholic acid competitively and reversibly inhibits EphA2–ephrin-A1 binding. A. In displacement studies, LCA was able to dose-dependently inhibit biotinylated ephrin-A1-Fc ectodomain (50 ng/ml) binding to the immobilized EphA2-Fc ectodomain (1 µg/ml). No displacement was observed for CA, DCA, CDCA. **B.** In saturation studies, competitive behavior was demonstrated by analyzing curves of EphA2–ephrin-A1 binding in the presence of increasing concentrations of LCA. **C.** KD values obtained from the saturation curves were used to calculate Log [Dose Ratio-1] and graphed as a Schild plot. A slope between 0.8 and 1.2 is associated with competitive binding. Through the intersection of the interpolated regression line with the X axis the pKi was estimated. **D.** Reversibility of LCA binding on EphA2 was obtained by incubating 200 µM of the compound and then washing some wells three times with PBS [Giorgio et al., 2011].



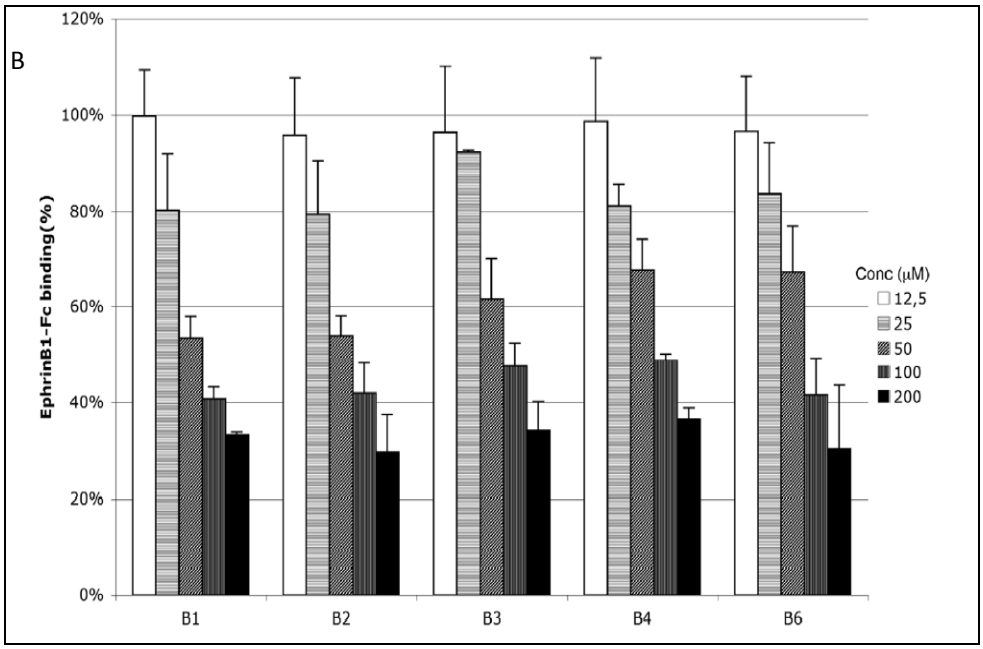


Figure 11: Lithocholic acid does not discriminate between Eph receptor subclasses. LCA dose-dependent displacement of the binding between EphA receptors and biotinylated ephrin-A1 (**A**) and EphB receptors with biotinylated ephrin-B1 (**B**). Data are the means of at least three independent experiments ± standard errors [Giorgio et al., 2011].

1.2 LCA inhibits Eph-kinase phosphorylation

Functional studies performed on PC3 cells showed the ability of LCA to inhibit EphA2 phosphorylation when the receptor was stimulated with 0.25 µg/ml ephrin-A1-Fc. Consistently with binding studies only LCA and not the other bile acids tested (CA, DCA, CDCA; data not shown) dose-dependently blocked the phosphorylation of the receptor showing an IC_{50} value of 48 µM in PC3 cells [Fig.12 A]

Since LCA was a promiscuous ligand for both EphA an EphB subclasses, the compound was tested also toward EphB4 receptor using T47D breast cancer cells. LCA was also able to antagonize in a dose-dependent manner the EphB4 receptor when stimulated by 3 µg/ml ephrin-B2 preclustered with 0.3 µg/ml Fc, with an IC_{50} value of 141 µM [Fig.12 B]. None of the compounds tested showed agonistic properties for either EphA2 or EphB4 (data not shown).

An MTT assay was also performed to demonstrate that the compound concentrations tested were not cytotoxic (data not shown).

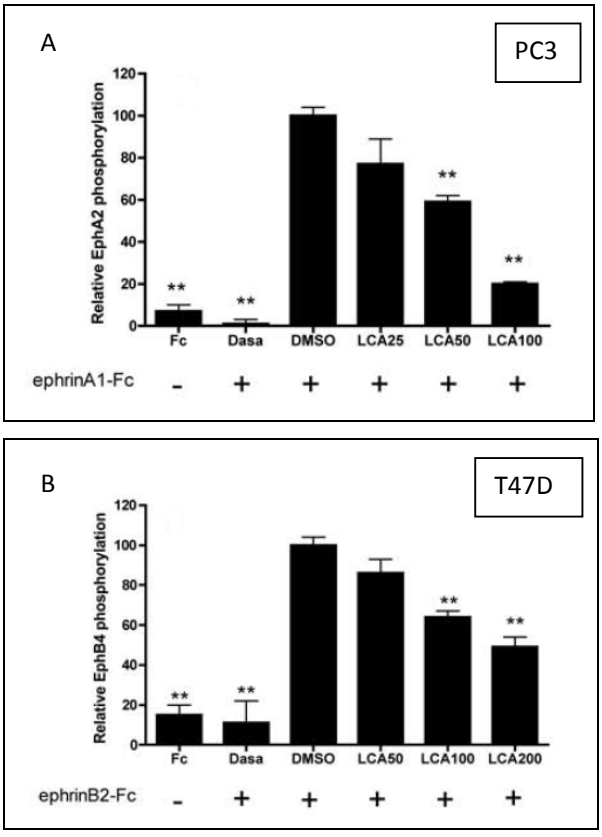


Figure 12: LCA antagonizes Eph-ephrin phosphorylation. LCA was able to dose-dependently inhibit **A.** EphA2 phosphorylation in PC3 cells stimulated by 0.25 µg/ml ephrin-A1 and **B.** EphB4 phosphorylation in T47D cells stimulated with 3 µg/ml ephrin-B2 preclustered for 1 hour with 0.3 µg/ml Fc. LCA was incubated for 20 minutes before ephrin stimulation for an additional 20 minutes. The DMSO concentration was 1%. Dasatinib at 1µM was used as reference compound. Phospho EphA2/EphB4 levels are relative to ephrin-A1/ephrin-B2-Fc +DMSO. Data are the means of at least three independent experiments ± standard errors. One-way ANOVA followed by Dunnet's post test was performed comparing ephrin-A1-Fc (A) and ephrin-B2 (B) to all the other columns. *p<0.05, **p<0.01 [Giorgio et al., 2011].

1.3 LCA does not affect the Eph kinase domain

An enzyme-based assay (LANCE detection method [Olive, 2004]) was performed in order to analyze the effects of LCA on the Eph kinase domain. In this assay, a substrate (Ulight-TK peptide at 50 nM) is phosphorylated when recombinant EphA2 kinase is activated, and a Europium-labeled anti-phospho-antibody recognizing the phosphorylated peptide emits light. Incubation of EphA2 with LCA at 100 µM did not modify the enzymatic activity of the receptor, suggesting that the LCA inhibitory effect observed in cells is due to inhibition of ephrin binding to the LBD of the receptor [Fig. 13].

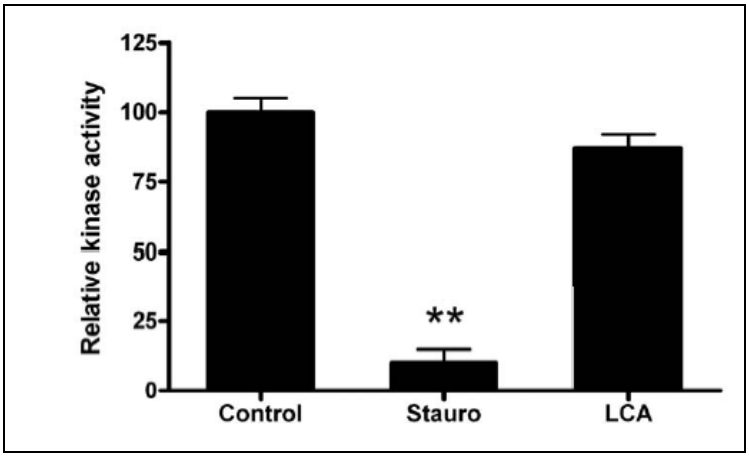


Figure 13: Lithocholic acid does not modify EphA2 enzymatic activity. Recombinant human EphA2 was incubated with 100 µM LCA, 1 µM staurosporine or 1% DMSO as a control for 30 minutes. The kinase activity was evaluated with the LANCE method using ATP and Ulight-TK peptide. A T-test was performed comparing LCA and staurosporine to the control column **p<0.01 [Giorgio et al., 2011].

1.4 LCA antagonism is specific for the Eph-ephrin system

Since LCA is known for its ability to play many physiological functions mainly related to metabolism and to interact with some receptors such as TGR5 and FXR [Kawamata et al., 2003; Makishima et al., 1999], we asked whether LCA can interact with other kinases besides EphA2. Therefore, the effect of LCA on other kinases (EGFR, VEGFR, IGFR1β and IRKβ) was tested in cellular functional assays. LCA at 100 µM was completely inactive against EGFR, VEGFR, IGFR1β and IRKβ, being unable to inhibit or stimulate the activity of these systems [Fig.14 A, Fig. 14 B].

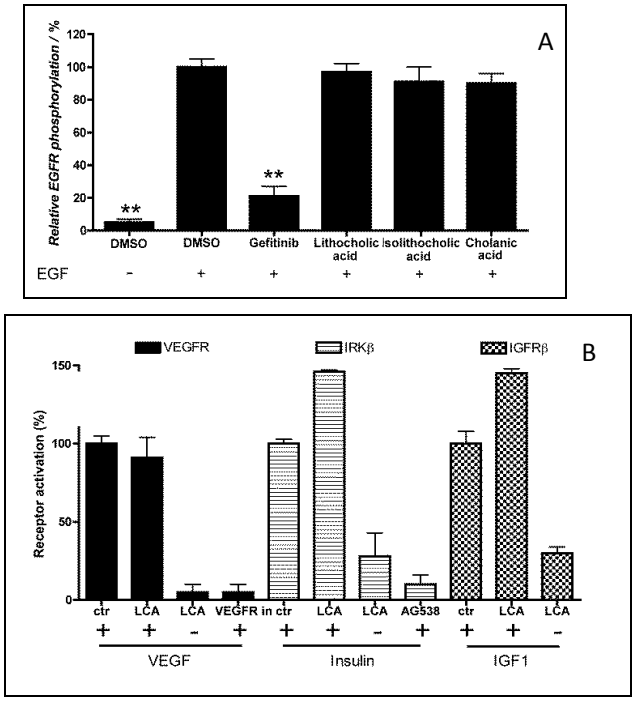


Figure 14: LCA does not affect EGFR, VEGFR, IRKβ, IGFRβ activity. A. In PC3 cells, LCA does not inhibit EGFR phosphorylation induced by 10 ng/ml EGF incubated for 20 minutes. Cells are pretreated with 100 µM LCA or 1% DMSO for 20 minutes. Gefitinib at 10 µM was used as reference compound. Phospho EGFR levels are relative to EGF+DMSO. Data are the means of at least three independent experiments ± standard errors. A T-test was performed for the comparison of PBS to LCA and EGF+DMSO to EGF+LCA and EGF+Gefitinib, **p<0.01 **B.** HUVE, HEPG2, A431 cells are stimulated 10 minutes with 0.1 nM VEGF, 5 nM IGF1 or 100 nM insulin, respectively, in presence of 100 µM LCA. Relative specific inhibitors are used. Data are the means of at least three experiments ± standard errors. A T-test was performed comparing ctr to other column of the same receptor, **p<0.01 [Giorgio et al., 2011].

2. Hit to lead

2.1 LCA binds EphA2 and not ephrin-A1

Many strategies aimed at interfering with the Eph-ephrin system have been explored over the years, and identification of low molecular weight ligands is one of the best ways for the production of new drugs.

The discovery of LCA as a small molecule able to interact with the Eph-ephrin system is an important starting point for the development of new compounds with higher affinity and potency as potential new drugs that could be employed for therapy.

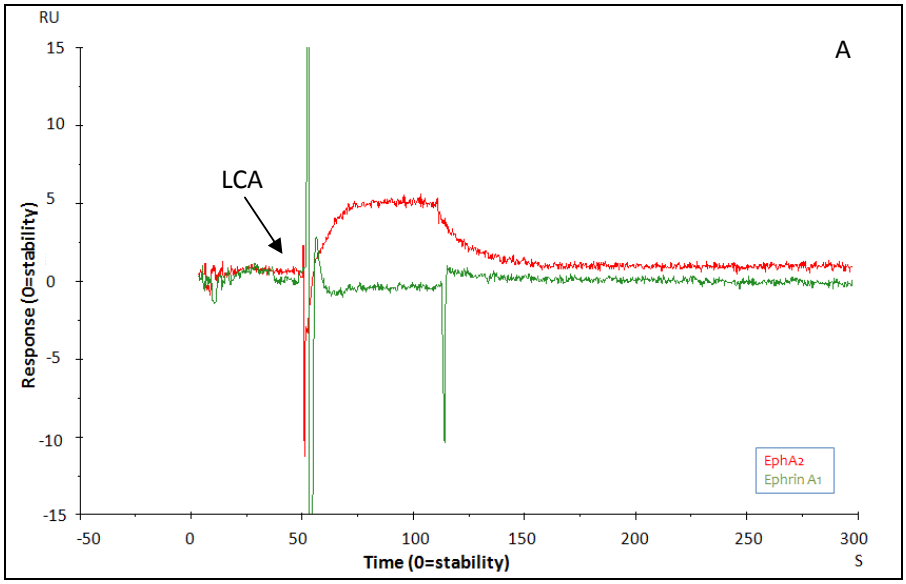
In our findings, LCA is a novel, reversible, competitive antagonist of Eph receptors that acts with specificity to inhibit the binding of ephrin ligands to the Eph receptor extracellular domain without affecting the receptor's kinase domain. This mechanism of action makes it more selective than kinase inhibitors.

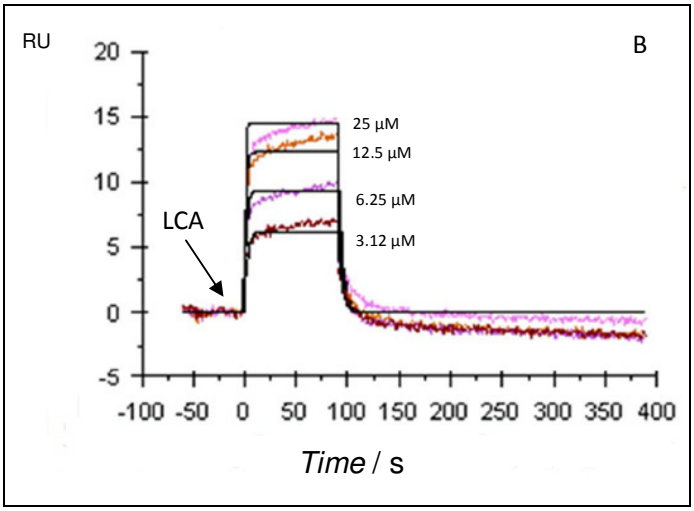
After its identification, we asked if LCA interfered with EphA2–ephrin-A1 binding by interacting with EphA2 or with ephrin-A1. The certain proof of the binding between LCA and Eph receptors came from SPR (Surface Plasmon Resonance) data, through Biacore Technology [Giannetti et al., 2008], performed by Prof. Luisa Bracci and Dr.Barbara Lelli of the Biotecnology Department of University of Siena. The compound was solubilized and incubated on a surface previously coated with EphA2-Fc or ephrin-A1-Fc. The surface was linked to optical biosensors [Pini et al., 2007], which respond to changes in mass: when a molecule binds the surface coated with the protein: mass variations are detected by sensors and the signal is recorded through a sensogram. In this assay, incubation of the EphA2 receptor with LCA induced a dose-dependent increase of the SPR signal, measured in RU (Resonance Units) [Fig. 15 B], while

incubation of ephrin ligands (ephrin-A1 and ephrin-B1) with LCA revealed no signal (ephrin-B1 data not shown) [Fig. 15 A].

A K_D value of 5.7 µM for LCA could be extrapolated from the sensogram, using a steady-state-analysis [Fig. 15 C] performed by plotting the binding at equilibrium versus the ligand concentration and assuming that the K_D equals to the concentration that yields 50% of the maximum response [Nuñez et al., 2012].

Considering these results, the LCA structure can be used as a model to design and characterize new EphA2 ligands endowed with better pharmacodynamic features.





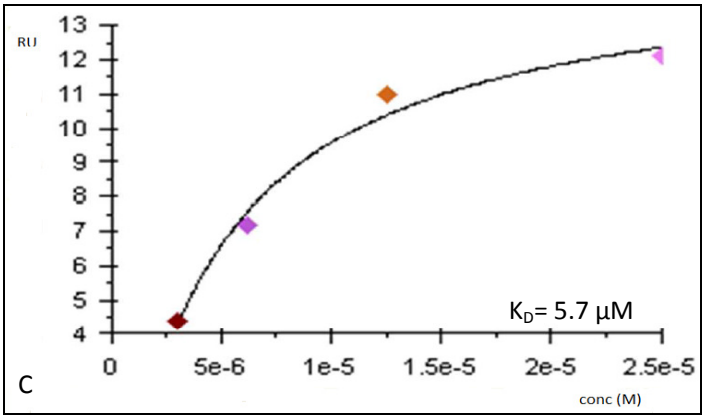


Figure 15 : LCA binds to the EphA2 receptor and not ephrin-A1. A. Incubation of ephrin-A1 with 50 µM LCA did not result in binding. **B**. Incubation of immobilized EphA2 receptor with different concentrations of LCA (3.12-25 µM) induced a dose-dependent binding of the compound with the receptor. **C.** Through the steady-state analysis we obtained a K_D value for LCA of 5.7 µM.

2.2 Identification of the pharmacophoric scaffold

Molecular modeling studies conducted by the group of Pharmaceutical Chemistry in the University of Parma, analyzed the positioning of the LCA scaffold ((5β)-cholan-24-oic acid) in the ephrin-binding pocket of the EphA2 receptor, allowing structure-activity relationship (SAR) predictions. Starting from the model, a rational series of 17 LCA derivatives, commercially available or obtained by chemical synthesis, were chosen and characterized for their ability to inhibit EphA2–ephrin-A1 binding [Tabel 1]. Some of these results regarding a first series of LCA derivatives have been recently published [Tognolini et al., 2012].

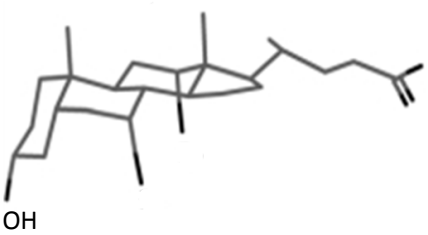
Ki values indicating the potency for inhibition of EphA2–ephrin-A1 interaction [Table 1] revealed that LCA derivatives were particularly sensitive to changes in the cyclopenta[a]perhydrophenantrene scaffold. An α-hydroxy group in -7 (R_2) or -12 (R_4) (compounds **2** (CA), **3** (DCA) and **4** (CDCA)) or the introduction of -6 (R_3) or -7 keto group (compounds **5** and **6**) led to inactive compounds.

If the α-hydroxy group in -3 (R_1) was oxidated (compounds **7** and **8**) or acetylated (compound **9**) the compounds had lower affinity than LCA (compound **1**) but they were still active, whereas, the inversion of the chiral center at position -3 led to isolithocholic acid (compound **10**), a compound more active than LCA. By contrast, sulfonation of the hydroxy group at -3 (compound **11**) was detrimental for the activity.

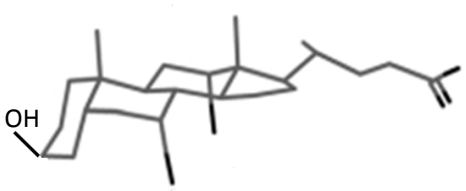
Modifications at the opposite end of the LCA hydrophobic core, where the acidic chain was positioned, led to different results: esterification (compound **12**), conjugation with amine derivatives (compounds **14** and **15**) or reduction to alcohol (compound **16**) were detrimental for activity, whereas conjugation of the carboxylic acid with a glycine (compound **17**) generated a LCA amino acid derivative conserving the potency of LCA.

Lastly, removal of α-hydroxy group resulted in a dramatic increase in activity yielding the most potent compound of the series, cholanic acid (compound **12**), which showed a Ki value of 5.1μM.

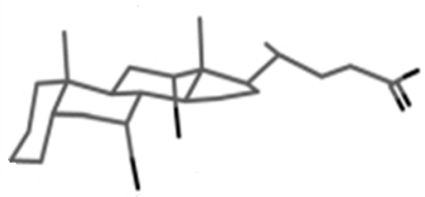
The best compounds of this series, isolithocholic acid and cholanic acid, were further characterized, in binding and functional assays in order to examine their effects *in vitro*



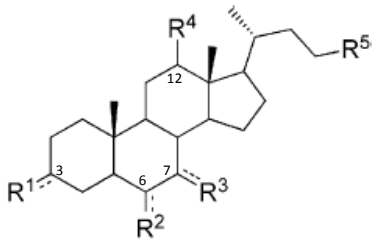
Lithocholic acid

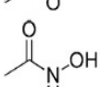
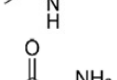
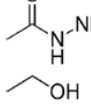
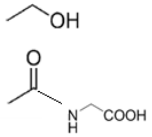


Isolithocholic acid



Cholanic acid



Compound	R1	R2	R3	R4	R5	Ki (μM)[a]
1	HO	H	H	H		49 ± 3.0
2	HO	H	HO	HO		> 200
3	HO	H	H	HO		> 200
4	HO	H	HO	H		> 200
5	HO	H	O	H		114 ± 13
6	HO	O	H	H		138 ± 20
7	O	H	H	H		157 ± 47
8	O	O	H	H		114 ± 14
9		H	H	H		88 ± 11
10	HO	H	H	H		25 ± 4.0
11		H	H	H		> 200
12	H	H	H	H		5.1 ± 1.4
13	HO	H	H	H		> 200
14	HO	H	H	H		> 200
15	HO	H	H	H		> 200
16	HO	H	H	H		186 ± 27
17	HO	H	H	H		38.5±0.09

Tabel 1: Structure-activity relationship for LCA derivatives. ([a]):Values are the means of at least three independent experiments ± SEM.

2.502.1 Cholanic acid and isolithocholic acid inhibit EphA2–ephrin-A1 binding

Among the series of 17 analogues analyzed, three had higher potency than **LCA** (compound **1**) for inhibition of EphA2–ephrin-A1 binding: **isolithocholic acid** (compound **10**), **cholanic acid** (compound **12**) and **glycolithocholic acid** (compound **17**). Leaving aside for a moment glycolithocholic acid, which will be discussed later, we can focus our attention on the other two compounds, isolithocholic acid and cholanic acid, whose characterization has been recently published [Tognolini et al., 2012].

In the studies performed, both compounds dose-dependently displaced biotinylated ephrin-A1 from the immobilized EphA2 with IC_{50} values of 67 µM for isolithocholic acid and 9.6 µM for cholanic acid [Fig.16 A].

We further analyzed the mechanism of binding through saturation curves [Fig.16 B, Fig.16 D] of EphA2–ephrin-A1 binding in presence of increasing concentrations of the two compounds, in order to calculate the K_D or the apparent K_D for each curve and draw the Schild plot, where log [DR-1] is the function of $-\log_{10}$ [inhibitor] [Arunlakshana et al., 1959]. We obtained well-interpolated regression lines (r^2 = 0.98 and r^2 = 0.99 for isolithocholic acid and cholanic acid, respectively) with slopes of 0.96 and 0.98, indicating competitive binding. The pKi resulting from the intersection of the interpolated line with the X axis showed values of 4.60 for isolithocholic acid (Ki = 25 µM) and 5.19 for cholanic acid (Ki = 5.1 µM) [Fig.16 C, Fig.16 E].

In order to examine the reversibility of binding, isolithocholic acid and cholanic acid were tested in displacement studies by incubating immobilized EphA2 with 100 µM of both compounds for 1 hour, then washing some of the wells before adding 50 ng/ml biotinylated ephrin-A1. Displacement of ephrin-A1 was detected only in unwashed wells, suggesting reversible binding to the EphA2 receptor (data not shown).

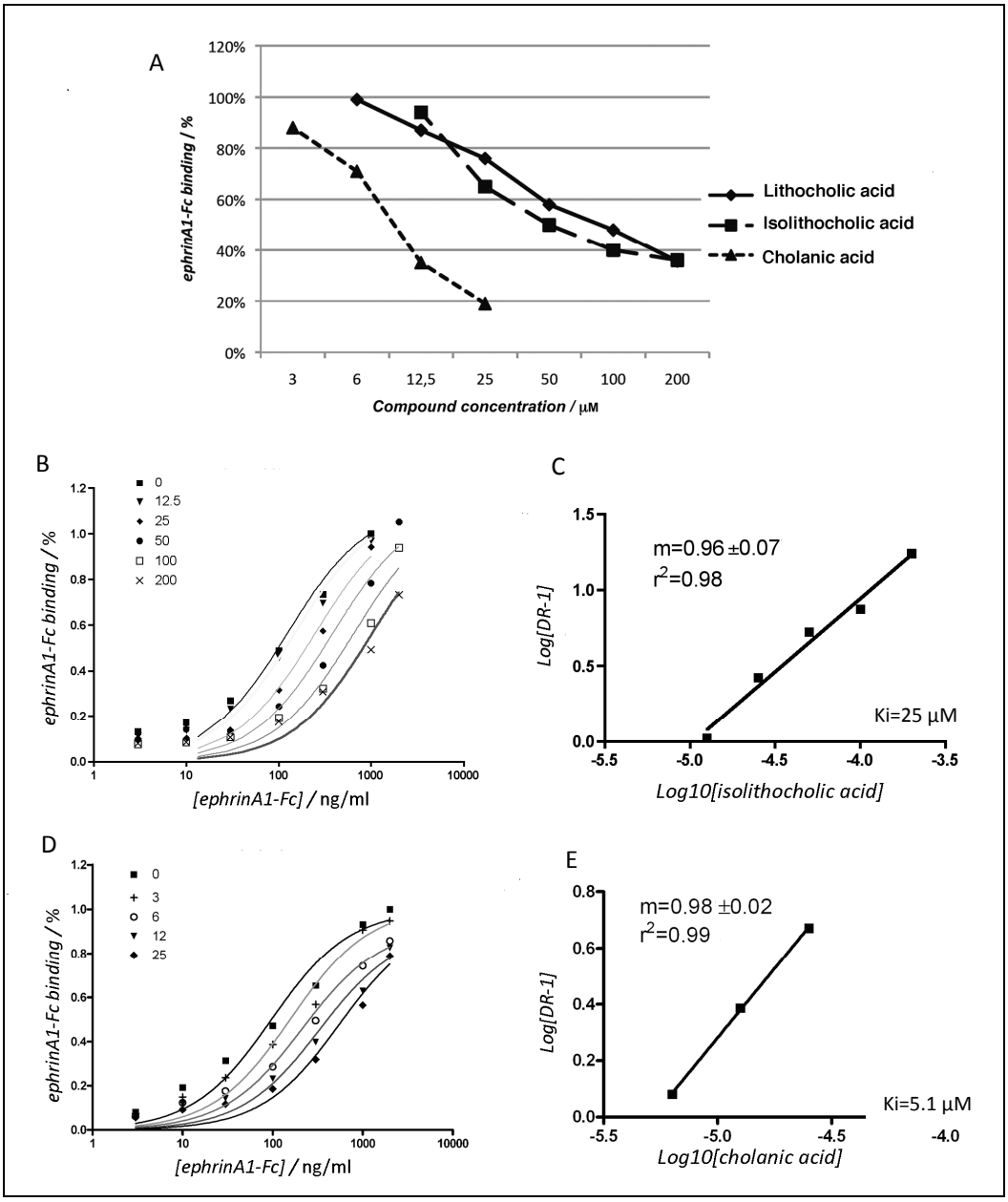
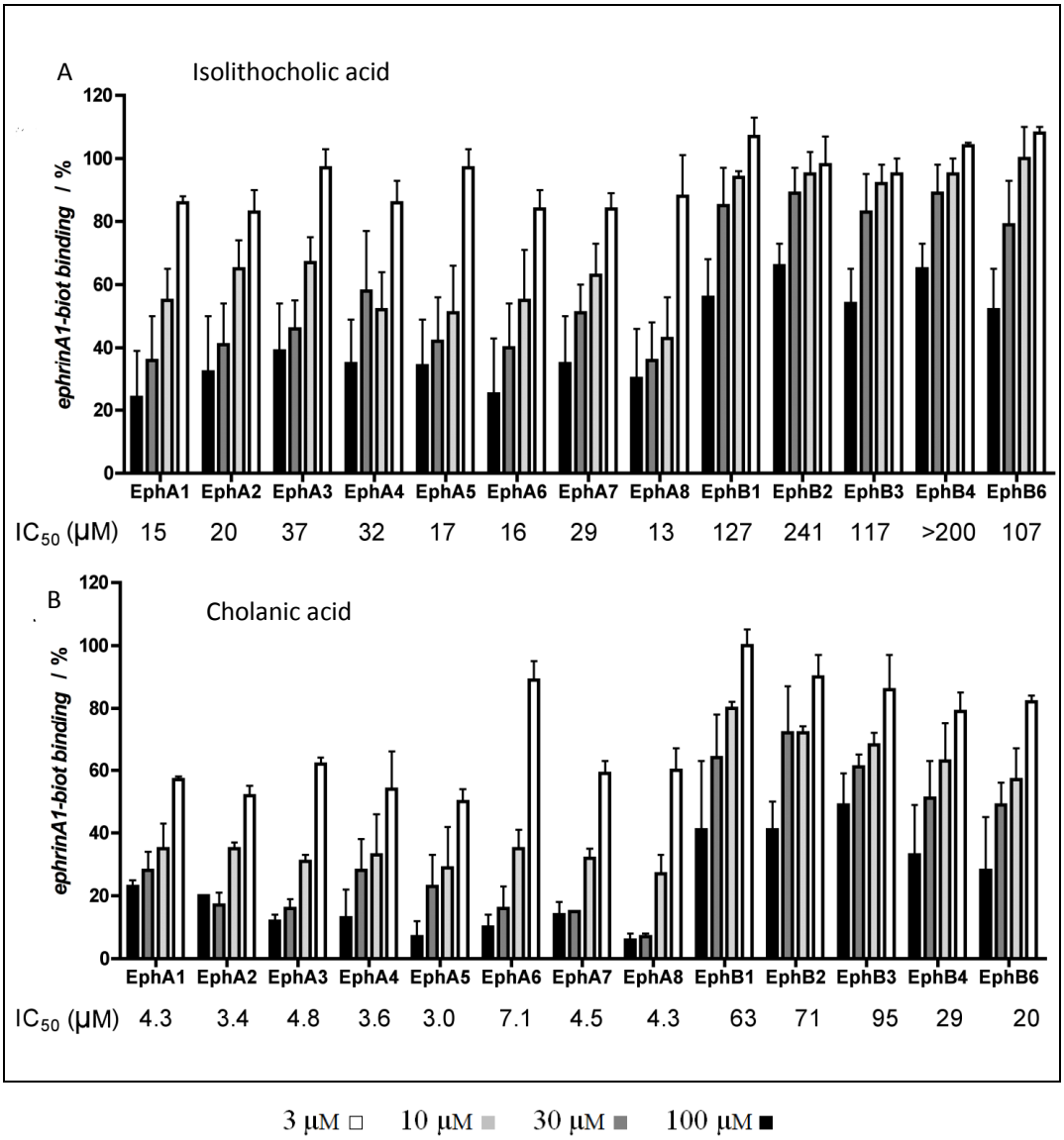


Figure 16: Isolithocholic acid and cholanic acid competitively displace EphA2–ephrin-A1 binding. A. Isolithocholic and cholanic acid showed higher potency than LCA in displacement of ephrin-A1 from the immobilized EphA2 receptor. **B.**, **D.** EphA2–ephrin-A1 binding in the presence of different concentrations of isolithocholic (0-200 µM) and cholanic acid (0-25 µM). **C.**, **E.** K_D values obtained from the saturation curves in **B., D.** were used to calculate log[DR-1] and extrapolate the Schild plots for the two compounds. The slope obtained indicates a competitive binding and the intersections with the X-axis represents the inhibition constant (Ki) values for isolithocholic and cholanic acid [Tognolini et al., 2012].

Moreover, isolithocholic acid and cholanic acid were more selective for the EphA receptor subclass in contrast to LCA, which is a promiscuous ligand. We analyzed the

ability of the compounds to inhibit ephrin binding to all EphA and EphB receptors using biotinylated ephrin-A1-Fc or biotinylated ephrin-B1-Fc respectively, at their K_D concentrations. Both showed higher potency in the inhibition of EphA receptors, especially cholanic acid, which showed IC_{50} values for EphA receptors 3-30 times lower than the IC_{50} values for EphB receptors [Fig. 17].



Fgure 17: Isolithocholic acid and cholanic acid discriminate between EphA and EphB subclasses. Isolithocholic acid (**A.**) and cholanic acid (**B.**) dose dependently displaced biotinylated ephrin-A1 or ephrin-B1 from immobilized EphA-Fc or EphB-Fc receptors, respectively. IC_{50} values are the means of at least three independent experiments; error bars represent standard errors [Tognolini et al., 2012].

2.2.2 Surface plasmon resonance (SPR) binding analysis of cholanic acid and isolithocholic acid to Eph receptors

Binding of cholanic acid to the EphA2 receptor was further investigated through SPR analysis in order to evaluate the mechanism of interaction. SPR sensograms showed the binding between different concentrations of cholanic acid [Fig. 18 A] and isolithocholic acid [Fig. 18 B] to the immobilized EphA2 receptor. The binding was saturable and specific. From the steady-state analysis K_D values of 1.16 µM and 3.9 µM for cholanic and isolithocholic acid, respectively, were obtained [Fig. 18 C, Fig. 18 D]. Moreover, the cholanic acid-EphA2 interaction was reversible, since the complex readily dissociated upon washing restoring the baseline signal.

SPR analysis was used again to confirm the selectivity of cholanic acid for the EphA subclass compared to the EphB subclass. The compound, tested at 6 µM, did not detectably bind to the EphB1 receptor while binding to the EphA2 receptor was readily detectable (data not shown).

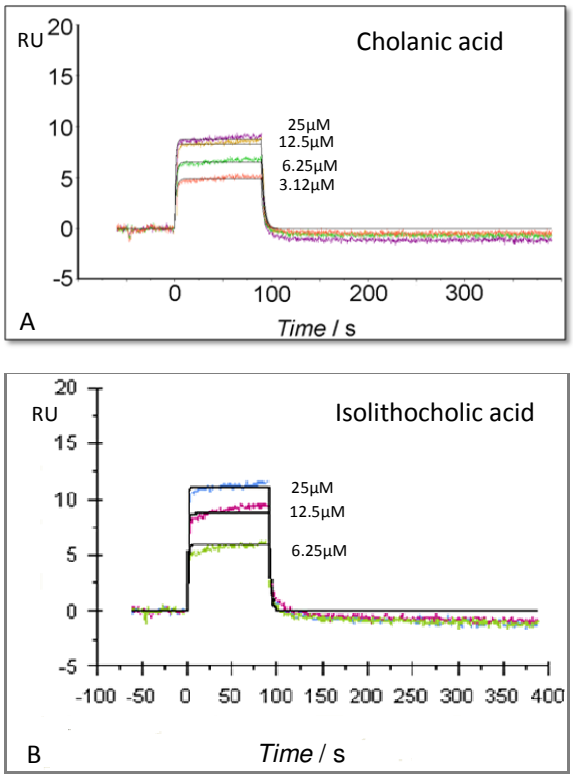
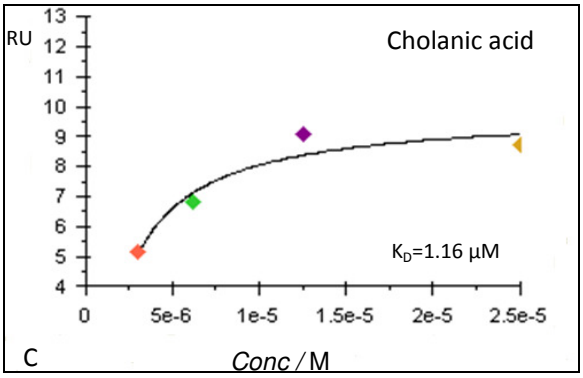
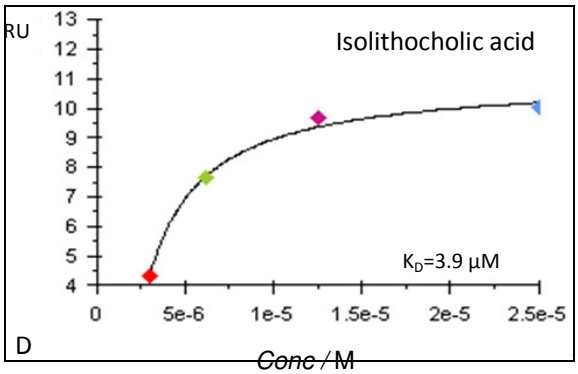


Figure 18 A and B: Cholanic and isolithocholic acid dose-dependently bind to the EphA2 receptor. Compounds were solubilized and incubated on the SPR surface previously coated with EphA2-Fc. The resulting sensograms showed a dose-dependent increase of the signal, measured in Resonance Unit (RU). After washing, the signal returned to base-line, indicating the reversibility of binding. Colored lines were used to denote different compounds concentrations (3.12-25 µM) [Cholanic acid sensogram was taken from Tognolini et al., 2012].

Figure 18: Steady-state analysis of cholanic (C) and isolithocholic acid (D). Steady-state curves were obtained by plotting the amount of binding at equilibrium (measured in Resonance Units) versus compound concentrations (M) and assuming that the K_D values correspond to the concentrations that produce 50% of the maximum response. We obtained K_D values of 1.16 µM for cholanic acid and 3.9 µM for isolithocholic acid.





2.2.3 Isolithocholic acid and cholanic acid inhibit Eph receptor phosphorylation at not-cytotoxic concentrations

In order to evaluate the effect of the compounds on receptors phosphorylation we performed functional assays with PC3 prostate cancer cells, which endogenously express EphA2, and T47D cells for the evaluation of EphB4. None of the compounds showed agonistic properties: similar to LCA, they were not able to activate the receptors. Instead, both isolithocholic acid and cholanic acid dose-dependently inhibited EphA2 and EphB4 phosphorylation induced by ephrin-A1-Fc or ephrin-B2-Fc, respectively [Fig. 19 A, Fig. 19 B] without cytotoxicity at the concentrations tested [Fig. 20].

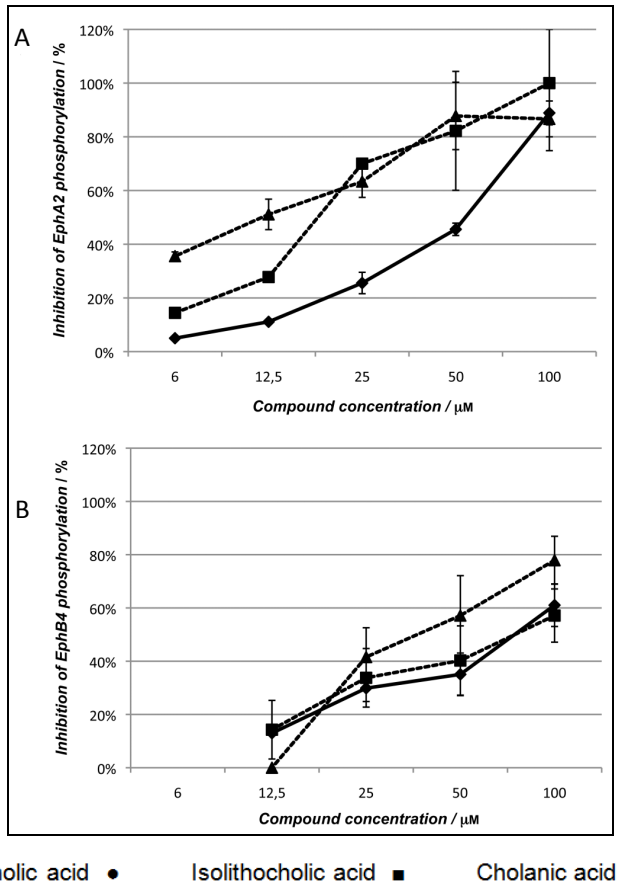


Figure 19: Lithocholic, isolithocholic and cholanic acid inhibit Eph receptors phosphorylation. The compounds dose-dependently inhibited EphA2 (**A**) and EphB4 (**B**) phosporylation induced by 0.25 µg/ml ephrin-A1-Fc or 3 µg/ml ephrin-B2-Fc preclustered with 0.3 µg/ml Fc, respectively. Cells (PC3 cells for EphA2, T47D cells for EphB4) were pretreated for 20 minutes with the compounds (or 1% DMSO) and then stimulated for an additional 20 minutes with the appropriate ephrin-Fc ligands (or Fc control). Dasatinib at 1 µM was used as reference compound to inhibit receptor phosphorylation (data not shown). Data are the means ± standard errors of at least three independent experiments [Tognolini et al., 2012].

Surprisingly, isolithocholic acid, which structurally differs from LCA only in the inversion of the hydroxy group at -3, showed higher potency than LCA with IC_{50} values of 17 µM for EphA2 and 71 µM for EphB4. This suggested an additional inhibitory effect of the compound on the intracellular kinase domain (see below [Fig. 21]).

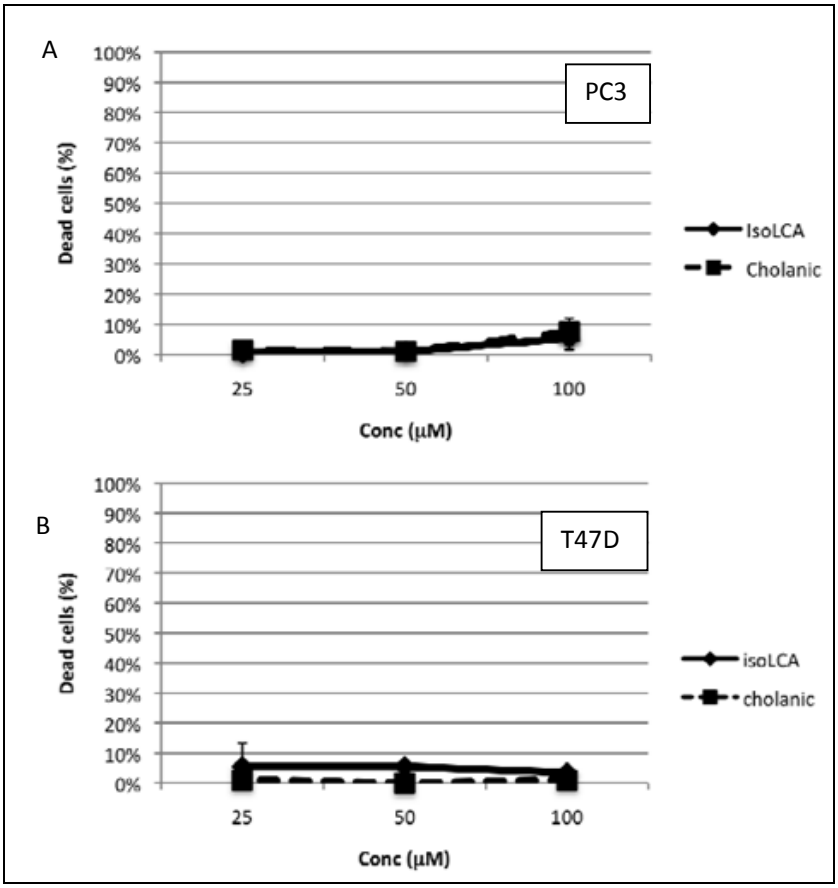


Figure 20: Isolithocholic and cholanic acid inhibit receptor phosphorylation at not cytotoxic concentrations. Compounds where incubated for 2 hours at 25-100 µM concentrations with PC3 cells (**A**) or T47D cells (**B**), then released LDH levels were measured. DMSO at 0.5% and Triton at 0.5% were used to determine the 0% and 100% toxicity, respectively. Data are the means ± standard errors of at least three independent experiments [Tognolini et al., 2012].

2.2.4 Cholanic acid does not inhibit EphA2 kinase activity

An enzyme-based assay (LANCE method, see above) was performed to evaluate the effect of cholanic acid on the receptor intracellular kinase domain. The purified EphA2 kinase domain was incubated in presence of 50 nM Ulight-TK peptide substrate, with or without 100 µM compound, and the levels of phosphorylated peptide were detected with a Europium-labeled antiphosphotyrosine antibody. Similar to LCA, cholanic acid did not affect the activity of the intracellular kinase domain, confirming that the observed inhibition of Eph phosphorylation is due to inhibition of ephrin binding [Fig. 21].

On the contrary, isolithocholic acid was significantly able to inhibit EphA2 kinase activity [Fig. 21] and this could explain the interestingly low IC_{50} values in the inhibition of Eph receptors phosphorylation found in cellular assays [Fig. 19].

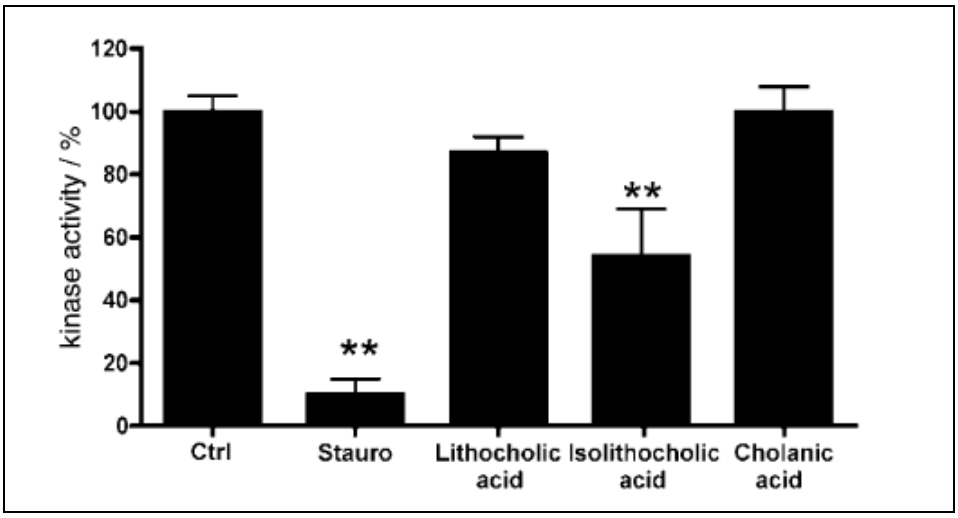


Figure 21: Cholanic acid does not target the EphA2 kinase domain. The recombinant EphA2 kinase domain was incubated for 30 minutes with 100 µM compounds or 1% DMSO as control. Kinase activation results in the phosphorylation of the peptide substrate, revealed through a specific Europium-labeled antibody. Staurosporine at 1 µM was used as reference compound able to completely inhibit kinase activity. **p<0.01 relative to control by one-way ANOVA, followed by Turkey's multiple comparison test [Tognolini et al., 2012].

2.2.5 Cholanic acid and isolithocholic acid antagonism is specific for Eph receptors

Similar to LCA, both cholanic acid and isolithocholic acid did not interfere with EGFR kinase activity. Compounds tested at the concentration of 100 µM in PC3 cells did not affect the activity of the EGFR activity when stimulated with EGF ligand [Fig. 22], indicating specificity for the Eph-ephrin system.

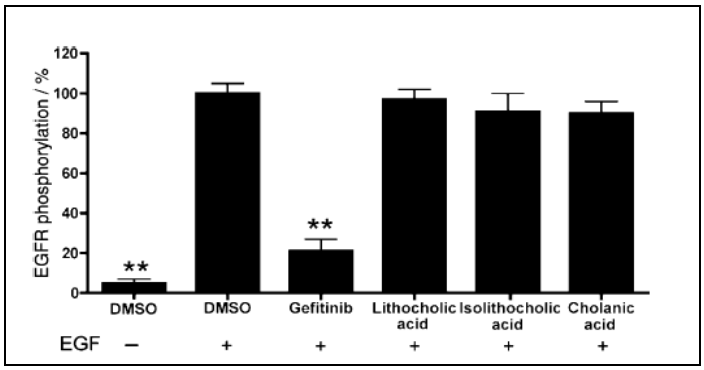


Figure 22: Cholanic acid and isolithocholic acid do not inhibit EGFR phosphorylation. PC3 cells were preincubated for 20 minutes with compounds at 100 µM or 1% DMSO as a control, then stimulated with 30 ng/ml EGF for 20 minutes. Gefitinib at 10 µM was used as reference compound. Phospho-EGFR levels are relative to the EGF+DMSO column. Data are the means ± standard errors of at least three independent experiments. **p<0.01 relative to EGF+DMSO by one-way ANOVA, followed by Turkey's multiple comparison test [Tognolini et al., 2012].

2.2.6 Cholanic acid and isolithocholic acid block EphA2-mediated PC3 cells retraction

In order to evaluate the antagonistic effect of cholanic acid and isolithocholic acid on cells a functional assay mediated by EphA2–ephrin-A1 interaction was performed. PC3 prostate cancer cells change their morphology when stimulated by exogenous ephrin-A1, since the interaction with EphA2 receptor induces cytoskeletal modifications that lead to cell rounding and a reduction in cell area.

Cholanic acid and isolithocholic acid inhibited EphA2-mediated cell rounding and retraction when stimulated by ephrin-A1-Fc, at concentrations up to 12.5 µM, reflecting the concentrations required for inhibition of EphA2 phosphorylation [Fig. 23]. None of

the compounds affected cell morphology in the absence of ephrin-A1 stimulation, confirming their lack of cytotoxicity.

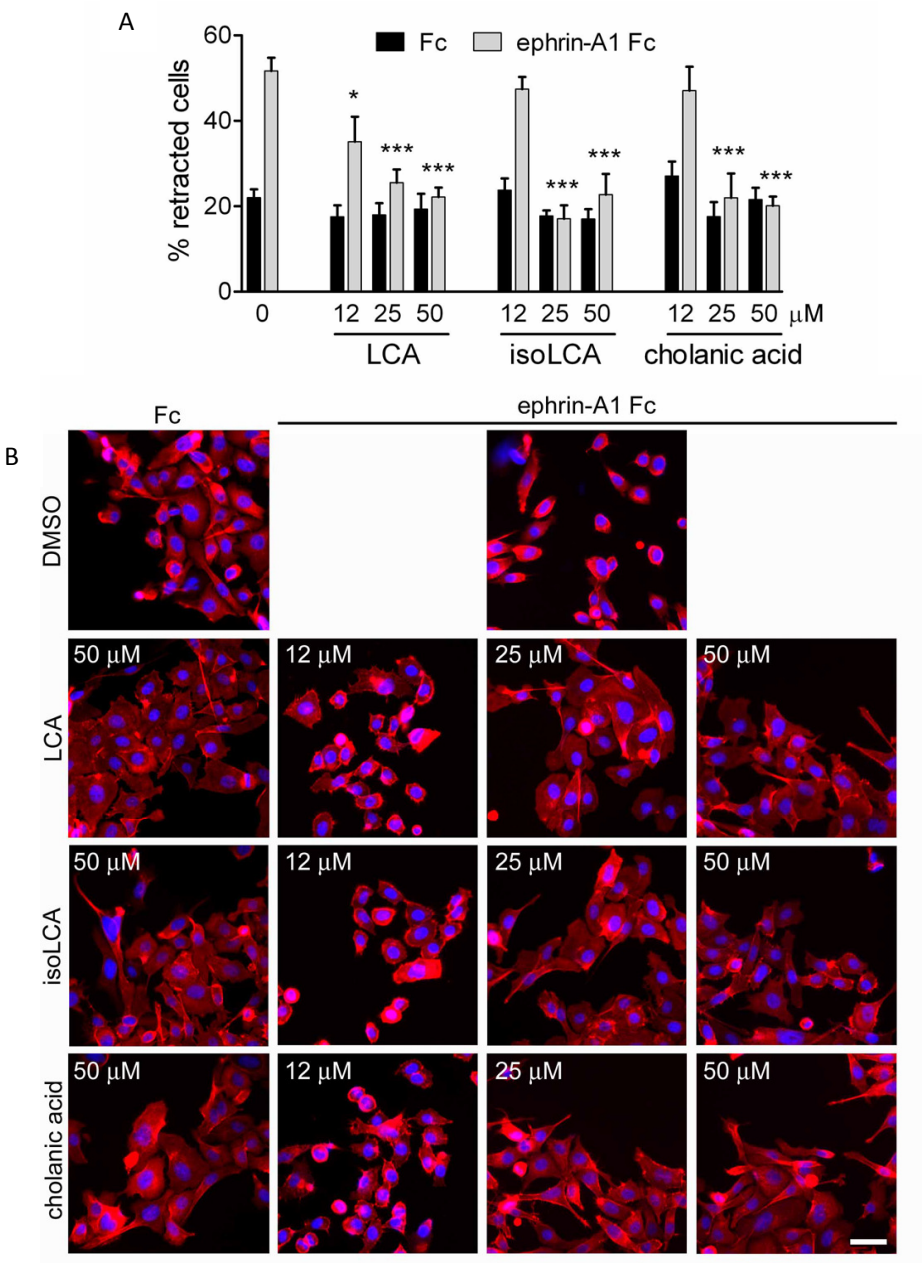


Figure 23: Lithocholic, isolithocholic and cholanic acid inhibit EphA2-mediated PC3 prostate cancer cells retraction. A. The compounds dose-dependently inhibited cell retraction after stimulation with ephrin-A1 (grey) while they did not affect the morphology of control cells incubated only with Fc (black). Columns show the average percentage of retracting cells. The percentage of cell retraction under various conditions was compared with that under Fc control conditions by one-way ANOVA and Dunnett's post test. **B.** Serum starved PC3 cells were pretreated with compounds at different concentrations (or 1% DMSO as a control) for 20 minutes and then stimulated with 0.5μg/ml ephrin-A1-Fc or Fc for 20 minutes. Then cells were then fixed with formaldehyde for 15 minutes and stained with rodhamine-phalloidin to label actin filaments (red) and DAPI to label the nuclei (blue) [Cholanic and isolithocholic acid data have been published in Tognolini et a., 2012].

2.3 LCA amino acid derivatives

2.3.1 Glycolithocholic acid

Another compound from the previous LCA derivatives series emerged for its activity: glycolithocholic acid (compound **17** in Table 1). This compound disrupted EphA2-ephrin-A1 binding with higher potency than LCA, showing a Ki value of 38.5 µM [Table 1].

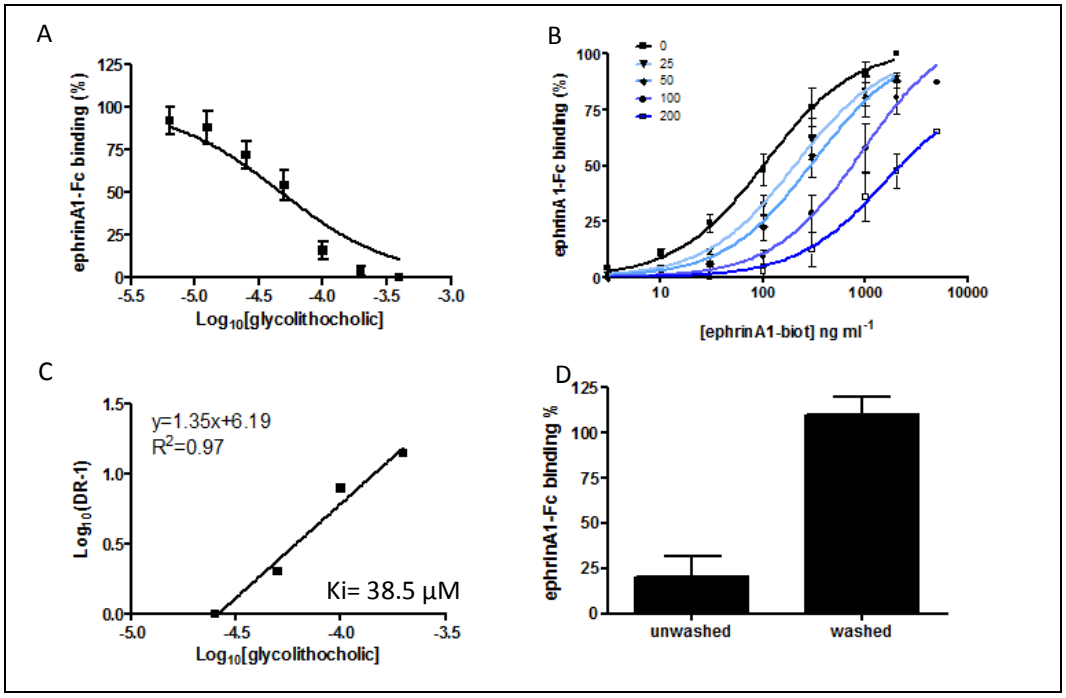


Figure 24: Glycolithocholic acid competitively disrupt EphA2–ephrin-A1 binding. **A.** Glycolithocolic acid dose-dependently displaced binding of the ephrin-A1-Fc ectodomain from the immobilized EphA2-Fc ectodomain. **B.** biotinylated ephrin-A1-Fc binding to the immobilized EphA2-Fc ectodomain in the presence of different concentrations of glycolithocholic acid. **C.** the dissociation constants (Kd) from the previous plot were used to calculate Log [Dose-ratio-1] and to graph the Schild plot. The intersection of the interpolated line with the X-axis yielded the pKi value. **D.** EphA2–ephrin-A1 binding in the presence of 100 µM of glycolithocholic acid with or without washing with PBS.

Accordingly to our pharmacophoric model, modifications of the LCA carboxylic group always generated inactive or poorly active compounds, because the receptor-ligand complex conformation is not favorable. Neverthless, the conjugation of the carboxylic group with glycine led to an active compound, with increased potency for inhibition of EphA2–ephrin-A1 binding compared to LCA. Binding studies showed that the glyco-conjugate was able to reversibly and competitively bind the EphA2 receptor in a dose-dependent manner [Fig. 24].

This finding indicates that conjugation of LCA with α-amino acids could be an alternative strategy to inhibit Eph receptors. Therefore, other modeling studies were performed to generate a new series of derivatives for further characterization.

2.3.2 Structure-activity relationship of amino acid derivatives

Based on these results, a new series of α-amino acids derivatives was synthesized and the compounds obtained [Table 2] were evaluated for their ability to disrupt the binding of ephrin-A1 to the EphA2 receptor through an ELISA binding assay screening [Giorgio et al., 2011].

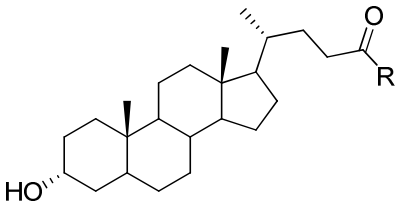
The pIC_{50} ($-\log_{10}$ [IC_{50}]) values obtained are shown in Table 2 with the relative standard error. LCA and glycolithocholic acid are included in Table 2 as reference compounds for the series and here they are named compound **1** and compound **2**, respectively, for convenience.

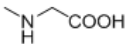
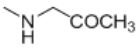
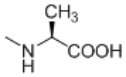
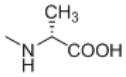
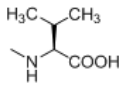
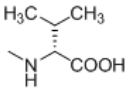
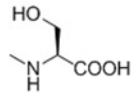
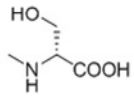
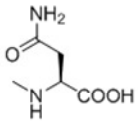
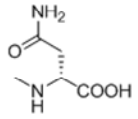
Compounds **1** and **2** were both active at preventing the binding of ephrin-A1 to EphA2, with pIC_{50} of 4.20 and 4.31, respectively. Conversely, compound **3**, the methyl ester derivative of **2**, was inactive, confirming the importance of a free carboxyl group for maintaining biological activity.

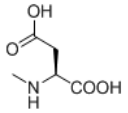
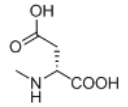
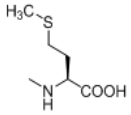
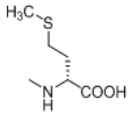
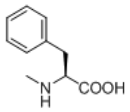
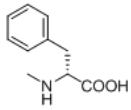
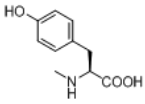
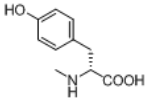
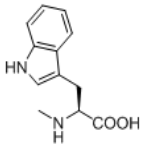
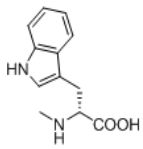
Analyzing the pIC_{50} values for these new derivatives it is evident that hydrophobic groups (compounds **4**-**7**) were better for the activity, regardless of the absolute configuration of the chiral centre of the amino acidic moiety. However, the introduction of hydrophilic groups was tolerated in case of side chains of limited size (compounds **8**, **9**), but if the side chains was bigger (compounds **10**, **11**) the activity was disrupt.

If conjugation of LCA with L- and D-Asp led to inactive derivatives (**12**, **13**), the introduction of amino acids carrying a lipophilic side chain yielded active compounds such as **14** and **15**, which had a methionine side chain, showing a limited increment in the binding activity compared to LCA.

The introduction of aromatic substituent had a dramatic impact on pIC_{50}. While compounds **16** and **17**, bearing a phenylalanine chain, resulted at least ten times more potent than LCA, the tyrosine derivatives (**18**, **19**) showed very low activity in EphA2–ephrin-A1 disruption maybe due to their reduced lipophilicity. The importance of the lipophilic group at α position was further confirmed by the tryptophan conjugates **20** and **21**, which are significantly more active than LCA. In particular, the L-Tryptophan conjugate (compound **20**) showed a pIC_{50} of 5.69 and therefore was the most potent compound of the series.



Compound	R	[a]pIC_{50}
1		4.24 ± 0.07
2		4.31 ± 0.09
3		< 3.50
4		4.70 ± 0.20
5		4.51 ± 0.09
6		4.62 ± 0.05
7		4.76 ± 0.11
8		4.48 ± 0.03
9		4.22 ± 0.09
10		<3.50
11		<3.50

Compound	R	[a]pIC_{50}
12		<3.50
13		<3.50
14		4.56 ± 0.10
15		4.56 ± 0.10
16		5.18 ± 0.12
17		5.12 ± 0.07
18		4.30 ± 0.16
19		4.00 ± 0.11
20		5.69 ± 0.12
21		4.69 ± 0.03

Tabel 2: Structure-activity relationship of amino acid conjugates: [a]): pIC_{50} values are the means of at least three independent experiments ± SEM.

2.3.3 A new competitive and reversible ligand for Eph receptors : L-Tryptophan LCA (L-Trp LCA)

Among the new α-amino acid derivatives tested the L-Tryptophan conjugate showed the highest potency for inhibition of EphA2–ephrin-A1 interaction. Therefore, this new Eph receptor antagonist was further characterized.

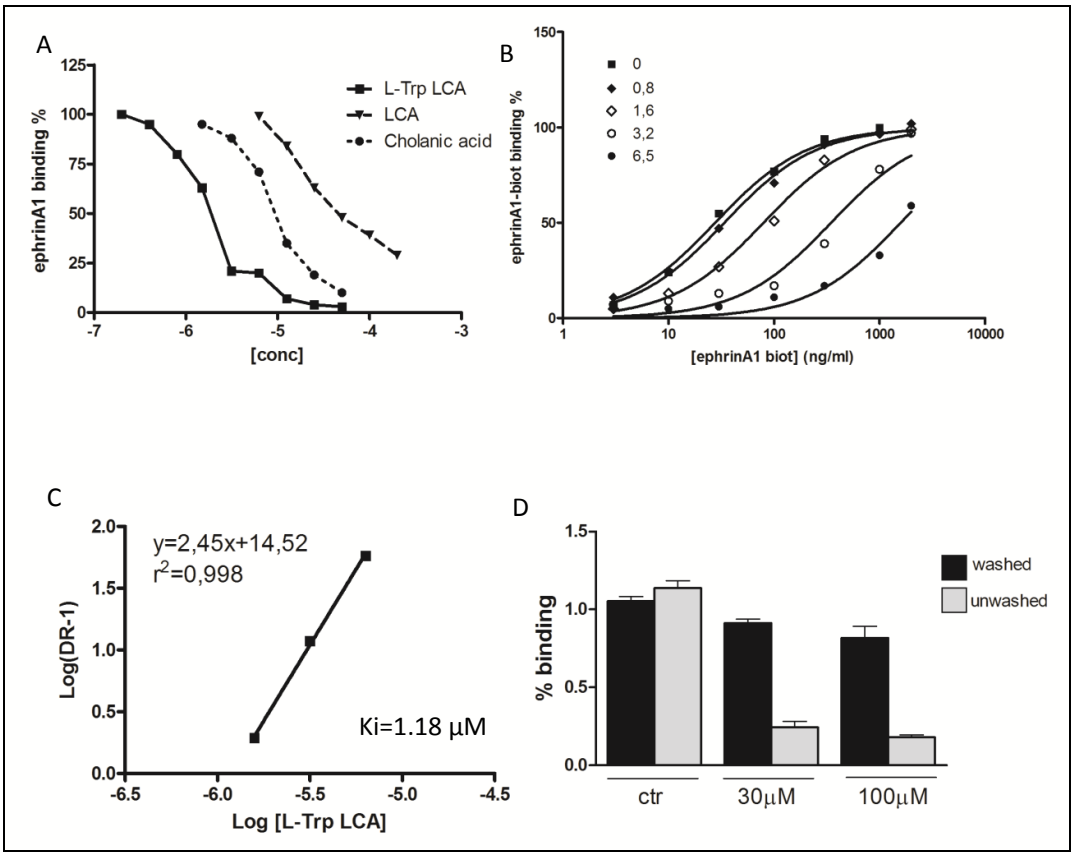


Figure 25: L-Trp LCA inhibit EphA2–ephrin-A1 binding. **A.** Comparison between displacement curves of L-Trp conjugate, LCA and cholanic acid. L-Trp LCA showed the best potency value among the derivatives tested. **B.** Saturation curves of biotinylated ephrin-A1 in presence of different concentrations (0.8-6.25 µM) of L-Trp LCA showed a competitive behavior**. C.** K_D values obtained from saturation curves in **B.** were used to calculate log[DR-1] and extrapolate the Schild plot. Through the intersection with the X-axis we obtained the inhibition constant (Ki) value of L-Trp LCA. **D.** EphA2–ephrin-A1 binding in presence of 30 µM and 100 µM L-Trp LCA with or without washing with PBS, indicating that the binding of the compound was reversible.

L-Trp LCA was first analyzed for its ability to disrupt EphA2–ephrin-A1 binding in an ELISA assay and the compound was able to dose-dependently displace biotinylated ephrin-A1 from the immobilized EphA2 with an IC_{50} value of 2.04 µM [Fig. 25 A].

The saturation curves for EphA2–ephrin-A1 binding in the presence of increasing concentrations of the compound [Fig. 25 B] were plotted and they pointed out a surmountable antagonism. The K_D or apparent K_D were calculated for each curve and the Schild plot was drawn. Through the interpolation of the regression line ($r^2 = 0.99$) a slope of 2.45 was obtained, indicating a stoichiometry ratio different from 1:1, suggesting another diverse mechanism of action of this compound compared with LCA or glycolithocholic acid. Finally, intersection of the interpolated line with X-axis gave a Ki value of 1.18 µM [Fig. 25 C]. L-Trp LCA, like LCA, cholanic and isolithocholic acid, also appeared to be a reversible binder when tested in displacement studies [Fig. 25 D].

.Furthermore, similar to previous compounds, L-Trp LCA was able to slightly discriminate between EphA and EphB subclasses, showing higher selectivity for the A subclass [Fig. 26].

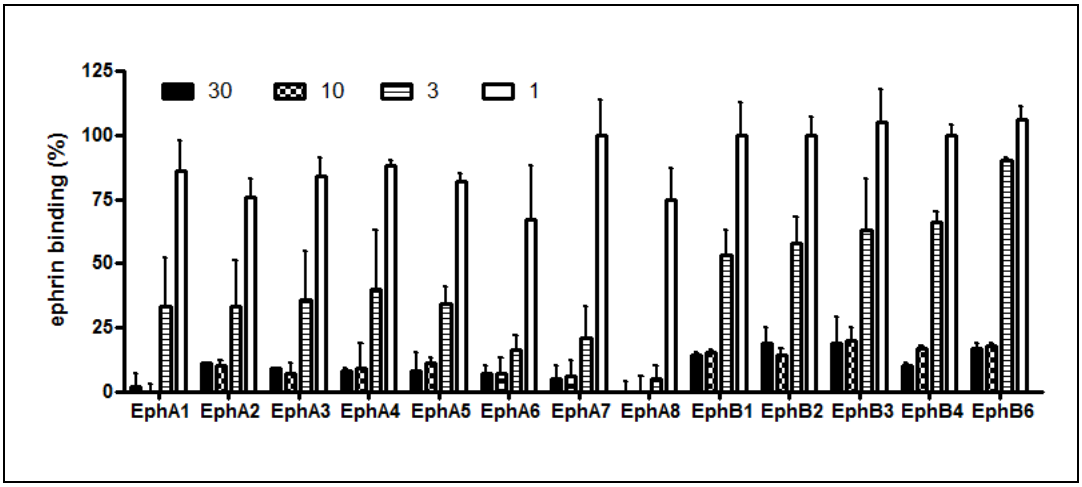


Figure 26: L-Trp LCA is selective for the EphA receptors subclass. The L-Trp conjugate was able to displace in a dose-dependent manner biotinylated ephrin-A1/B1 from immobilized EphAs-Fc or EphBs-Fc receptors, respectively. IC_{50} values are the mean of at least three independent experiments; error bars represent standard errors.

2.3.4 L-Trp LCA is an antagonist of the EphA2 receptor

Functional assays performed using the PC3 cell line revealed the antagonistic properties of the L-Trp derivative towards the EphA2 receptor. The compound showed good potency (IC_{50} value of 12.5 µM) in the inhibition of EphA2 phosphorylation following ephrin-A1 ligand stimulation [Fig. 27].

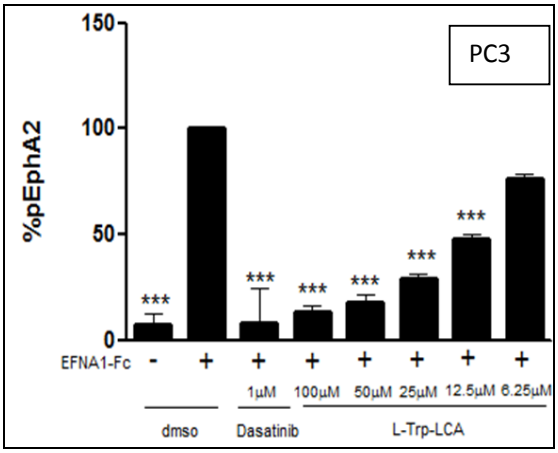
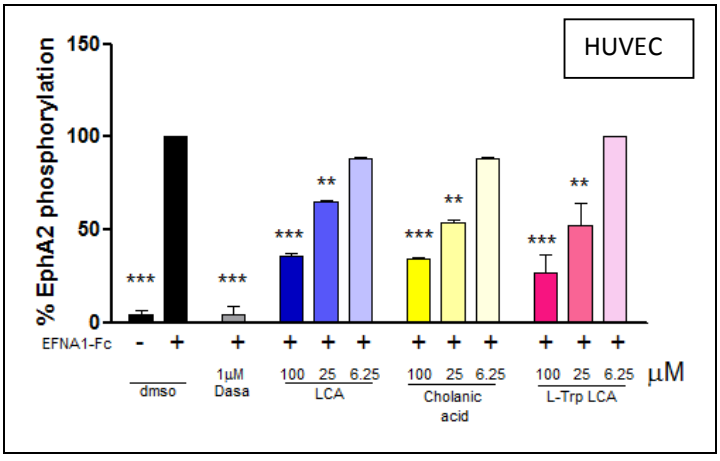


Figure 27: L-Trp LCA is able to LCA dose-dependently inhibit EphA2 phosphorylation in PC3 cells. EphA2 phosphorylation was induced by a 20 minute stimulation with 0.25 µg/ml ephrin-A1-Fc after pretreatment with the compound (or 1% DMSO). Dasatinib at 1 µM was used as reference compound and it completely blocked receptor phosphorylation. Shown are the means ± standard errors from at least three independent experiments. ***p<0.01.

In order to further characterize the L-Trp conjugate in angiogenic functional assays, we also investigated the activity of the compound in HUVE cells.

L-Trp LCA inhibited phosphorylation of the receptor induced by the ephrin-A1 ligand in HUVECs, although it showed lower potency than in PC3 cells [Fig. 28]. Further investigation will be necessary in order to determine the effect of L-Trp LCA on EphB phosphorylation.

Figure 28: L-Trp LCA is able to dose-dependently inhibit EphA2 phosphorylation on HUVE cells. Compound or 0.5% DMSO were incubated for 20 minutes before stimulation with 0.25µg/ml ephrin-A1. Dasatinib at 1µM was used as reference compound. The histogram shows means ± standard errors from at least three independent experiments, **p<0.05, ***p<0.01.



LDH assays were performed to measure the toxicity of L-Trp LCA in PC3 and HUVECs: the concentrations used in the inhibition of EphA2 phosphorylation were not cytotoxic for cells after incubation for two hours (for PC3 cells) [Fig. 29] or forty minutes (for HUVECs) [Fig. 30].

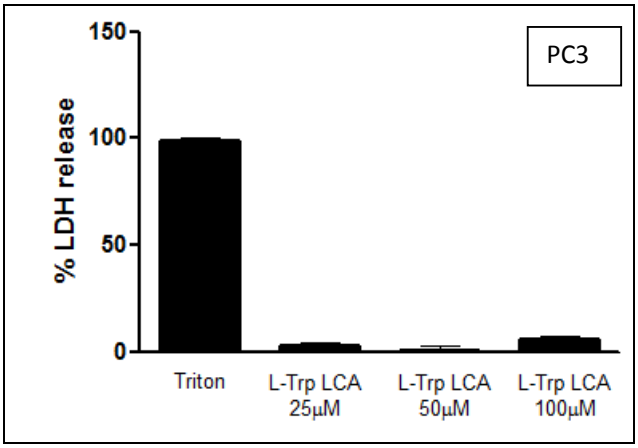


Figure 29: L-Trp LCA is not cytotoxic in PC3 cells. The compound was incubated in serum starved PC3 cells for two hours and LDH release was measured through a colorimetric reaction. Triton at 0.5% was used as a control (100% LDH release). Shown are the means of at least three independent experiments ± standard errors.

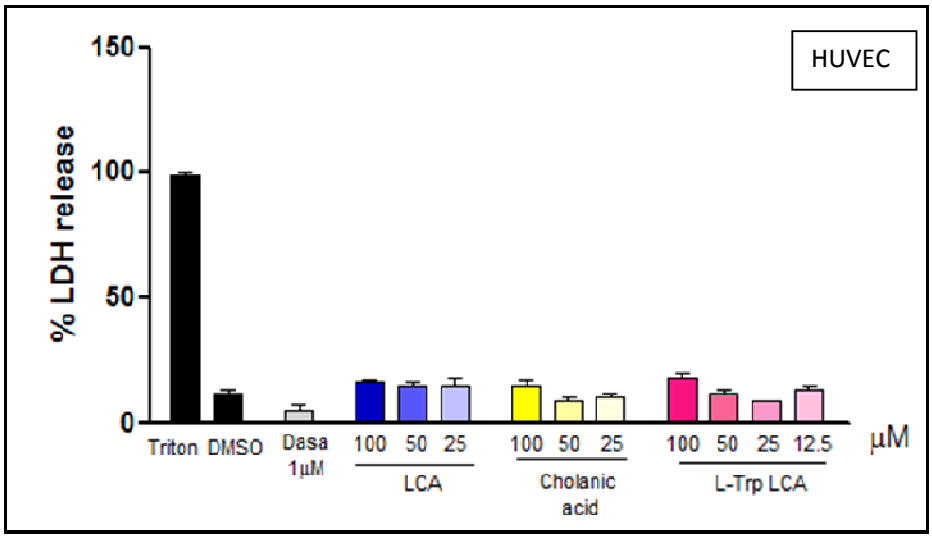


Figure 30: L-Trp LCA is not cytotoxic in HUVE cells. HUVECs were incubated with L-Trp LCA or the other compounds for 40 minutes, then LDH release was measured through a colorimetric reaction. LCA and cholanic acid showed a comparable not significant level of LDH release in HUVEC cells compared to L-Trp LCA. Triton at 0.5% was used as a control (100% LDH release). Shown are the means of at least three independent experiments ± standard errors.

2.3.5 L-Trp LCA does not affect EGFR phosphorylation

Similar to the other LCA derivatives tested, L-Trp LCA was specific for the Eph system and it did not interfere with the EGFR. In fact, at 100 µM the L-Trp conjugate did not affect the activity of the EGFR stimulated with EGF ligand in PC3 cells [Fig. 31].

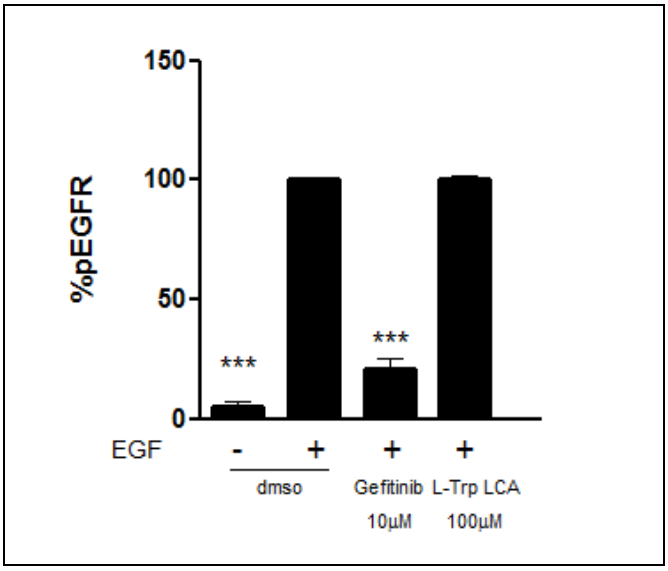


Figure 31: L-Trp LCA antagonism is specific for the Eph-ephrin system . After incubation of compound or 1% DMSO for 20 minutes , PC3 cells were stimulated with 10 ng/ml EGF. Gefitinib at 10 µM was used as reference compound. Shown are the means ± standard errors from at least three independent experiments. ***p<0.01.

2.3.6 L-Trp LCA inhibits EphA2-mediated PC3 cells retraction

LCA, cholanic and isolithocholic acids showed to be able in the inhibition of EphA2-mediated cell rounding when stimulated by ephrin-A1-Fc, at concentrations up to 12.5 µM.

Similarly, also L-Trp LCA was able to block PC3 cell retraction after stimulation with ephrin-A1-Fc confirming the antagonistic properties of the compound [Fig. 32]. Moreover L-Trp LCA did not affect cell morphology in absence of stimulation.

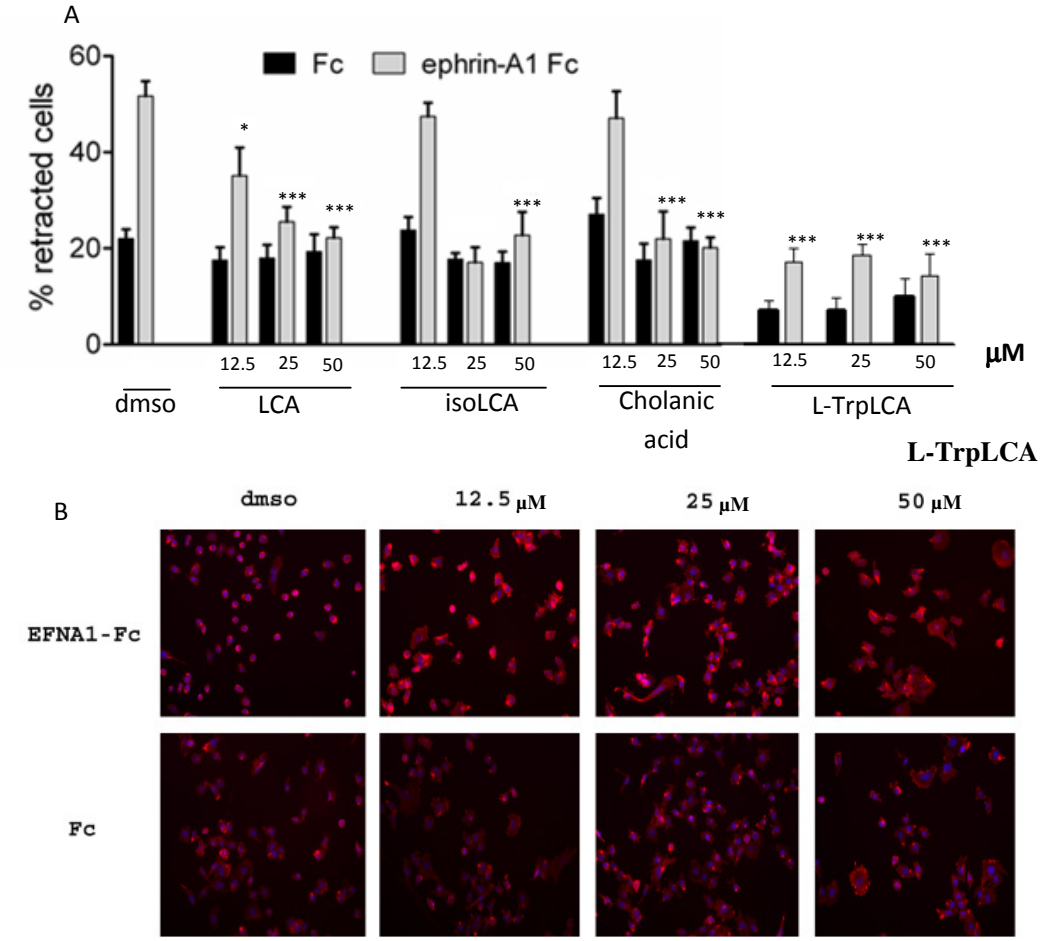


Figure 32: L-Trp LCA inhibits EphA2-mediated PC3 cells retraction and rounding A. L-Trp conjugate was able to inhibit in a dose-dependent manner cell retraction after stimulation with ephrin-A1 (grey), and it is more potent compared to the other active bile acids. It did not affect cell rounding in absence of ephrin-A1-Fc and incubating with Fc only (black). Columns show the average percentage of retracting cells. The percentages of cell retraction under various conditions were compared with those under Fc control conditions by one-way ANOVA and Dunnett's post test. *p<0.05; ***p<0.01. **B.** Serum starved PC3 cells were treated with different concentrations of L-Trp-LCA (12.5-50 µM) or 1% DMSO as control, for 20 minutes. Then cells were stimulated with 0.5 µg/ml ephrin-A1-Fc or Fc for 20 minutes. Cells were fixed and stained with rodhamine-phalloidin to label actin filaments (red) and DAPI to label the nuclei (blue).

2.3.7 L-Trp LCA blocks angiogenesis in HUVECs without cytotoxicity

Eph-ephrin system plays a role in pathological angiogenesis and interference with this process could be useful for the disruption of tumor microenvironment.
HUVECs, which consistently express Eph receptors [Pandey et al., 1995], are able to form a wide net of vessels when seeded in a layer of matrigel overnight so they represent a well estabilished model for angiogenesis assays.

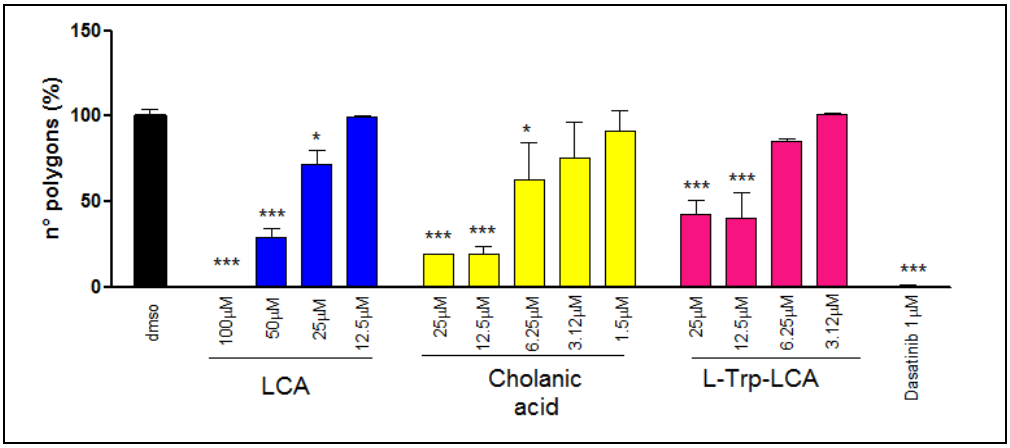


Figure 33: L-Trp LCA is able to inhibit HUVEC tube formation. Serum starved HUVECs were treated with compounds and immediately seeded on 24-well-plate precoated with a layer of Matrigel. After 15 hours cells were fixed and pictures were taken. All the compounds were able to inhibit in a dose-dependent manner vessels formation after 15 hours incubation. Dasatinib at 1 µM completely abolished the formation of the tubes. Columns show the average percentage of polygons counted. Data are the means of at least three independent experiments ± standard errors. One-way ANOVA followed by Dunnet's post test was performed comparing the DMSO column to all the other columns. ***p<0.01, *p<0.05.

L-Trp LCA was able to inhibit tube formation after 15 hours incubation with HUVECs at concentrations up to 12.5 µM [Fig. 33]. According to the phosphorylation data, also cholanic acid and LCA showed the ability to block vessels reticulation at not-cytotoxic concentrations [Fig. 34 B]. The multikinase inhibitor dasatinib was used as reference compound and it was able to completely disrupt the formation of the tubes at the concentration of 1 µM. The effect of dasatinib on HUVECs could be explained with its antiproliferative effect [Fig. 34 A], which reduced the viability of cells inducing the abolishment of vessels reticulation. Conversely, bile acids derivatives showed the inhibition of tube formation at concentrations not related to the antiproliferative effect

[Fig. 34 A]. Neverthless, the high potency values in the inhibition of angiogenesis in HUVECs suggested that probably Eph system is not the only target of LCA derivatives and that maybe compounds could interfere with other pathways. As a matter of fact, the angiogenetic process is the intricate result of a variety of different system signal cascades. Further investigations should be considered to understand the role of LCA derivatives in angiogenesis.

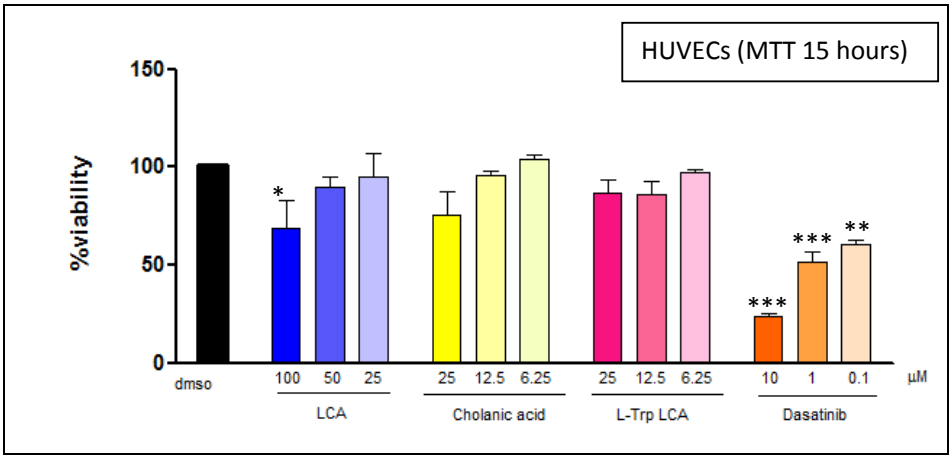


Figure 34 A: L-Trp LCA does not affect cell proliferation. L-Trp conjugate was incubated at different concentrations for 15 hours and MTT assay was performed to evaluate HUVEC cell viability. The compound showed no antiproliferative effects at concentrations up to 25 µM. Dasatinib, tested at concentration range of 0.1-10 µM, showed to inhibit cell proliferation after 15 hours. Data are the means of at least three independent experiments ± standard errors. *p<0.05, *** p<0.01.

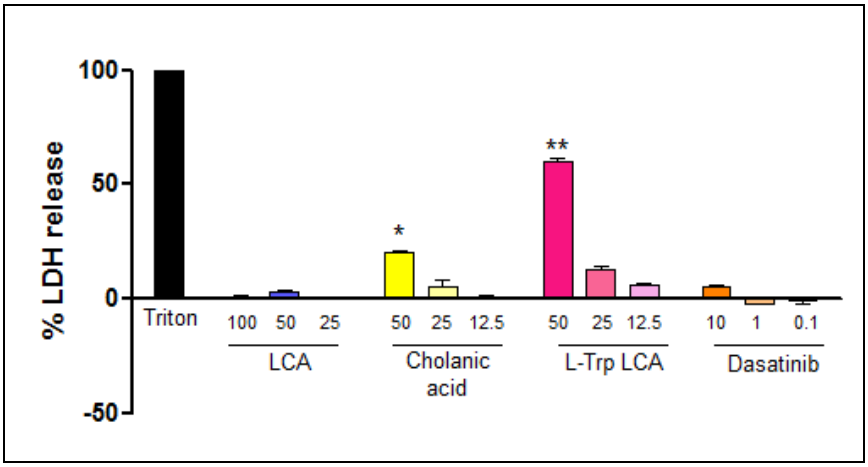


Figure 34 B: LCA derivatives cytotoxicity in HUVE cells. Compounds were incubated at different concentrations for 15 hours and LDH release was measured to evaluate the cytotoxicity. L-Trp LCA showed no cytotoxic effects at concentrations lower than 25 µM. None of the compounds at the concentrations tested in the tube formation assay were cytotoxic on HUVECs. Triton at 0.5% was considered as 100% LDH release, DMSO as 0%. LDH release. Data are the means of at least three independent experiments ± standard errors. **p<0.01, *p<0.05 relative to control by one-way ANOVA, followed by Turkey's multiple comparison test.

3. Polyphenols

We tested a variety of different molecules in our first ELISA assay based screening: drugs, bioactive endogenous molecules and also extracts and essential oils derived from plants. Among hundreds of molecules tested, beside LCA (which was widely characterized above) we focused our attention also on a group of natural plant extracts featuring by the presence of polyphenols.

Since the correlation between phytochemicals and and Eph-ephrin system was not completely known, we have chosen to screen the widest possible array of plant derivatives, including both lipophilic and hydrophilic extracts of edible plant known to contain flavonoids and other polyphenols, terpenes, terpenoids, glycosides and other secondary metabolites. Between 133 phytocomplexes screened, nine extracts showed an interaction with EphA2–ephrin-A1 binding, so they are here further characterized [Mohamed et al., 2011].

3.1 Nine extracts inhibit EphA2–ephrin-A1 binding

In order to identify phytocomplexes able to interfere with EphA2–ephrin-A1 binding an ELISA based assay screening was performed as previously described [Giorgio et al., 2011]. All the extracts and oils were screened at 5 mg/ml incubating them for 1 h before adding biotinylated ephrin-A1-Fc at a concentration corresponding to its K_D, then extracts displacing more than 40% binding were further studied.

Nine extracts (*A. uva-ursi, A. indica, E. ribes, G. biloba, L. speciosa, P.emblica, P. granatum, T. bellerica,* and *T. chebula*) merged in their ability to dose-dependently inhibit EphA2–ephrin-A1 binding with a potency in the µg/ml range. None of the essential and fixed oils of our library interfered with EphA2–ephrin-A1 binding, whereas the above-mentioned plant extracts completely abolished EphA2–ephrin-A1 interaction.

Displacement studies using different concentrations of extracts were performed in order to calculate the inhibitory concentration reducing binding of 50% (IC_{50}) [Fig. 35 A].

The extracts of *A. uva-ursi, A. indica, E. ribes, G. biloba, L. speciosa, P. emblica, P. granatum, T. bellerica,* and *T. chebula* dose-dependently inhibited EphA2–ephrin-A1 binding with IC_{50} values ranging from 0.83 µg/ml (*A. indica*) to 24 µg/ml (*E. ribes*) [Tabel 3].

The inhibition of binding was reversible: we repeated displacement incubating for 1 hour each extract at 20 µg/ml (with the exception of *E. ribes* incubated at 80 µg/ml being less potent), some wells were washed and displacement was detected only in not-washed wells [Fig. 35 B].

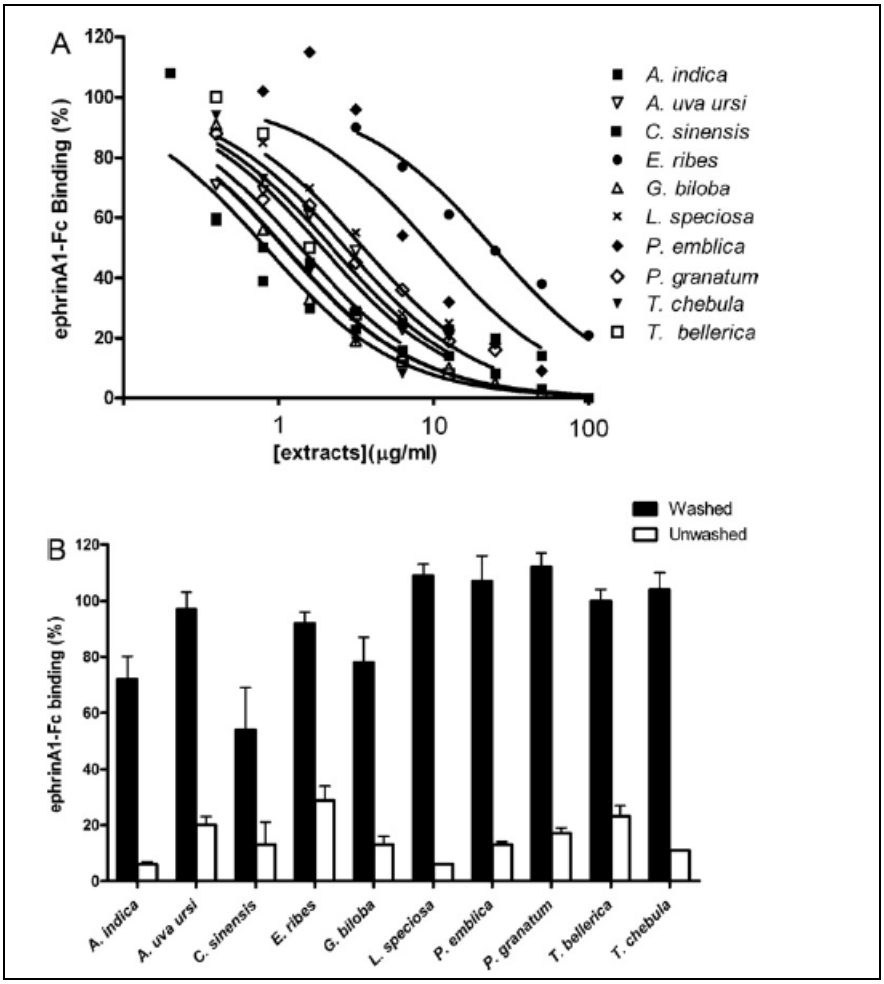


Figure 35: Nine extracts dose-dependently inhibit EphA2–ephrin-A1 binding. A. Displacement curves of the active extracts. *A.indica* showed to be the most potent between the phytocomplexes tested. Data are the means of at least three independent experiments ± standard errors. Curves were fitted using one-site competition non-linear interpolation with Prism 4.0. **B**. EphA2–ephrin-A1 binding in presence of 20 µg/ml of extract (80 µg/ml for *E.Ribes*) with or without washing three times with PBS [Mohamed et al., 2011].

3.2 Extracts inhibit EphA2 phosphorylation at not-cytotoxic concentrations

Functional studies were performed on PC3 cells in order to analyze the properties of the extracts screened, towards EphA2 receptor. All the extracts were able to dose-dependently inhibit EphA2 phosphorylation induced by 0.25 µg/ml ephrin-A1-Fc on PC3 cells showing IC_{50} values [Tabel 3] ranging from 0.31 µg/ml (*T. chebula*) to 11.3 µg/ml (*E. ribes*) [Fig. 36]. Green tea was used as natural reference, while dasatinib, a multikinase inhibitor, was used at 1 µM as reference compound since it is able to inhibit EphA2 kinase. On the other hand, none of the extracts was able to induce EphA2 phosphorylation on PC3 cells when incubated for 30 minutes at 40 µg/ml (data not shown), indicating that they were not agonist. MTT assay was performed to confirm that inhibitory effect showed by extracts were not due to any toxic effect on cells. None of the extracts decreased PC3 cell viability when incubated up to 40 µg/ml for 48 hours. Only green tea extract pointed out a slightly higher cytotoxicity when compared with other plants [Fig. 37].

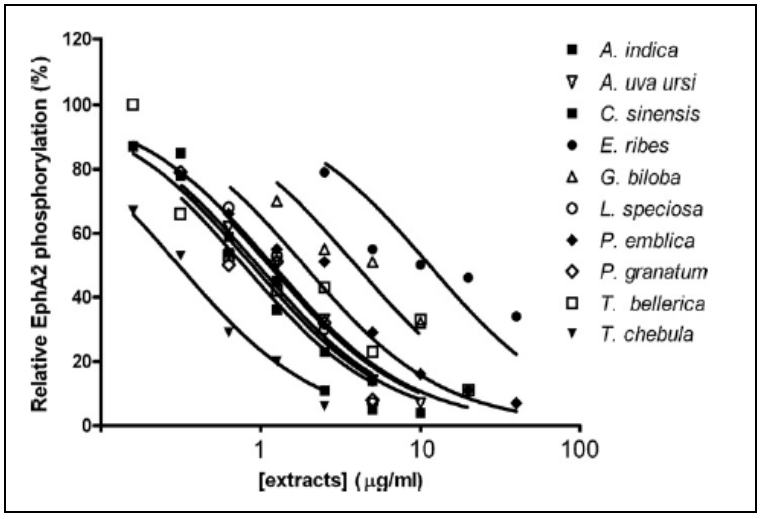


Figure 36: Extracts dose-dependently inhibit EphA2 phosphorylation. Serum starved PC3 cells were stimulated with 25 µg/ml ephrin-A1-Fc for 20 minutes before the pretreatment with extracts for 20 minutes. Green tea and Dasatinib at 1 µM were used as reference compounds. Phosphorylation levels of EphA2 are relative to ephrin-A1-Fc+PBS. Data are the means of at least three independent experiments . Curves were fitted with one-site competition non-linear regression using Prism 4.0 [Mohamed et al., 2011].

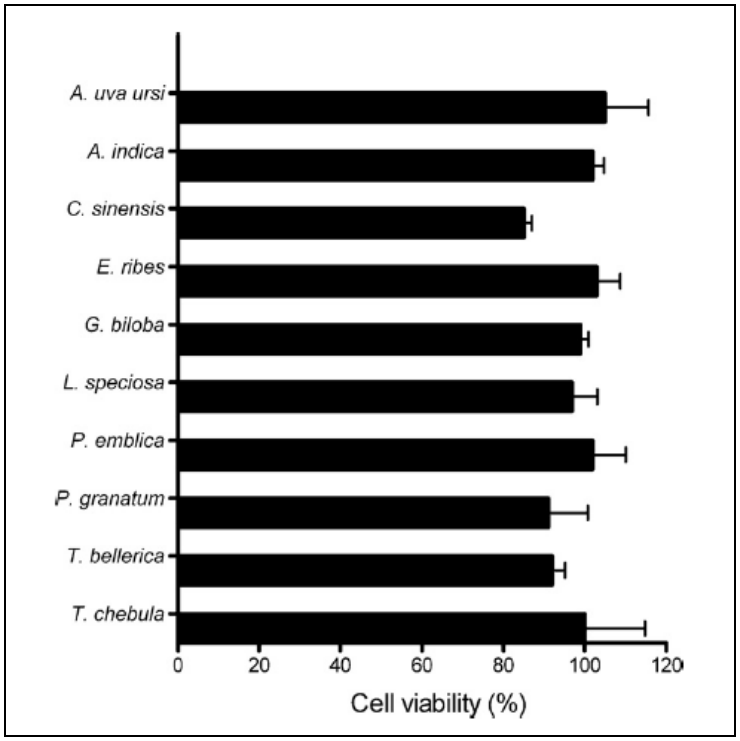


Figure 37: Extracts do not show cytotoxicity. PC3 cells were incubated with 40 µg/ml of each extract for 48 hours, then MTT was added at the concentration of 1 mg/ml for 2 hours and the resulted formazan crystals were solubilized with DMSO. The absorbance was read at 550 nm. Data are the means of at least three independent experiments ± standard errors [Mohamed et al., 2011].

	IC_{50} (95% confidence interval) (µg/ml)	
	Binding	EphA2-phosphorylation
Azadirachta indica	0.83 (0.49–1.4)	0.88 (0.73–1.1)
Ginkgo biloba	1.1 (0.75–1.6)	3.9 (2.4–6.4)
Camellia sinensis	1.1 (0.62–2.0)	0.78 (0.41–1.5)
Terminalia chebula	1.4 (0.65–2.9)	0.31 (0.24–0.38)
Terminalia bellerica	1.9 (0.93–3.9)	1.2 (0.63–2.2)
A. uva-ursi	2.2 (1.4–3.4)	1.1 (0.89–1.4)
Punica granatum	2.7 (2.0–3.7)	0.95 (0.58–1.5)
Lagerstroemia speciosa	3.5 (2.7–4.7)	1.2 (0.65–2.0)
Phyllanthus emblica	8.9 (4.9–16)	1.9 (1.4–2.5)
Embelia ribes	24 (19–30)	11.3 (6.0–21)
Dasatinib	Inactive	0.012 (0.008–0.018)

Tabel 3. Correlation between IC_{50} values for EphA2—ephrin-A1 binding and EphA2 phosphorylation inhibition of for the active extracts. Data are the means of at least three independent experiments ± standard errors [Mohamed et al., 2011].

3.3 Extracts do not interfere with EGFR activity

In order to evaluate the specificity of the effect exhibited by extracts, they were tested also on EGFR kinase. All active extracts showed to be inactive when tested towards EGFR phosphorylation, induced by 30 ng/ml EGF [Fig. 38]. The EGFR kinase inhibitor gefitinib used as reference compound at the concentration of 10 µM. Green tea extract (containing 44% EGCG) did not interfere with EGFR phosphorylation up to 5 µg/ml accordingly with previous observations in which EGCG provided a significant activity only above 50 µM (4.8 µM in our case) [Sah et al., 2006].

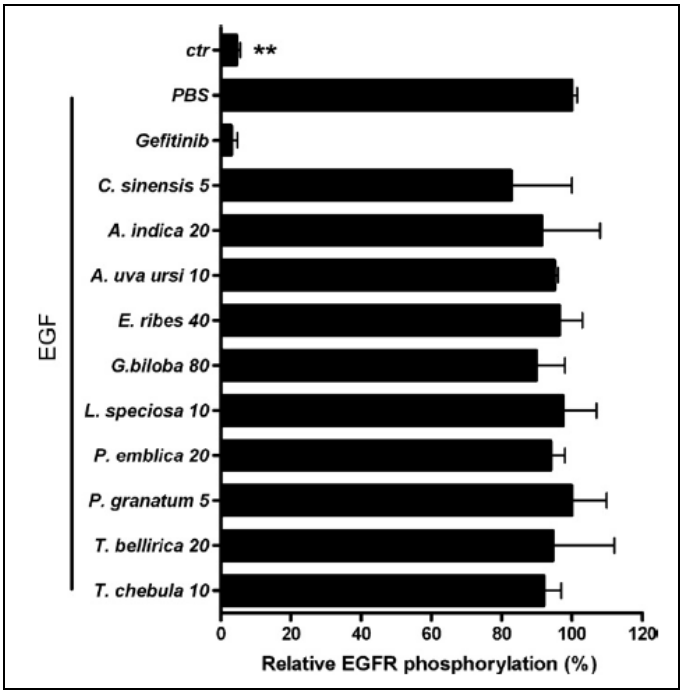


Figure 38: Extracts do not affect EGFR phosphorylation. Serum starved PC3 cells were incubated 20 minutes with the indicated concentrations (µg/ml) of extracts or PBS as control, then they were stimulated for 20 minutes with 30 ng/ml EGF. Gefitinib at 10 µM and green tea extracts were used as reference compounds. Phospho-EGFR levels are relative to EGF+PBS. Data are the means of at least three independent experiments ± standard errors. A T-test was performed comparing all the column to EGF+PBS, **p<0.01 [Mohamed et al.,2011].

DISCUSSION AND CONCLUSION

1. LCA and LCA derivatives

1.1 Discovery of a hit compound: LCA

Eph-ephrin system is involved in a variety of physiological and pathological processes, including cancer. Alterated expression of this system promotes tumorigenesis and cancer dissemination, especially overexpression of EphA2 and EphB4 receptors are the most involved in the development of aggressive and metastatic tumor phenotype.

Based on this evidence our aim was to identify small molecules able to interact with EphA2–ephrin-A1 activity through ELISA assay based screening and among hundreds of molecules tested we identify LCA, a natural secondary bile acid.

Bile acids have been considered for long time only as detergent molecules important for lipid solubilization and intestine absorption during digestion. Neverthless, a number of study demonstrated the role of bile acids as regulatory molecules: for instance, Katona and colleagues showed an enantiospecific apoptotic effect of bile acids enantiomers on colon cancer cells [Katona et al., 2009], or other papers described a specific interaction of bile acids with the nuclear Farnesoid X Receptor (FXR), involved in hepatic lipid and glucose metabolism [Makishima et al., 1999], and with G protein

coupled receptor TGR5, leading to the increase of cAMP in CHO cells [Kawamata et al., 2003].

Consistently, we discovered an interaction between a secondary bile acid, LCA and specific receptors: LCA was a competitive, reversible antagonist of Eph receptors, able to dose-dependently displace the binding between Eph receptors and ephrin ligands and it was also able to inhibit the phosphorylation of Eph receptors in functional assays.

For the first time we provided evidence of the interaction of LCA with Eph-ephrin system. First of all, we obtained proper displacement of saturation curves, a proper slope in a Schild Plot and the reversibility of binding. Moreover LCA was able to inhibit Eph receptor phosphorylation induced by ephrin ligand in different cell lines (PC3 and T47D) without affecting enzymatic activity, so targeting the extracellular ligand binding domain of receptors and not the intracellular kinase domain. Lastly, LCA did not interact with other kinase systems including EGFR, VEGFR, IRKβ and IGFRβ in cellular studies, demonstrating that the antagonism is specific for Eph-ephrin system.

Unlike the other natural bile acids (DCA, CDCA, CA), LCA was the only able to bind Eph receptors, and sole LCA was able to inhibit Eph phosphorylation after stimulation with ligands, and without cytotoxicity. Moreover the Ki of LCA towards EphA2–ephrin-A1 interaction was six time lower than its critical micelle concentration [Katona et al., 2007].

LCA did not discriminate between EphA and EphB receptor sublclasses, therefore this suggested an interaction with a highly conserved region essential for both EphA and EphB receptor binding to their physiological ligands. Ephrin ligands accommodates its G-H loop into the hydrophobic domain of receptor, so this have initially suggested us that LCA scaffold was able to fit in this hydrophobic channel of receptors (see below for the hypotesys of binding modeling).

The definitive proof of binding with Eph receptors came with the SPR analysis: LCA interfered with EphA2–ephrin-A1 binding by interacting with EphA2 and not with ephrin-A1. SPR analysis pointed out that first of all LCA was able to dose-dependently bind the receptor and also, that the binding was reversible upholding the evidence showed in the results above.

Starting from these observations and considering the role played by Eph-ephrin system in many different pathophysiological conditions, development of new compounds able to interact with higher affinity and potency with Eph-ephrin system was intriguing.

1.2 Hit to lead: LCA derivatives

The recent resolution of the three-dimensional structure complex of EphA2–ephrin-A1 through X-ray crystallography by Himanen and colleagues, led to a further investigation in the mechanism of binding of LCA to EphA2 receptor [Himanen et al., 2009].

The interaction between these EphA2 and ephrin-A1 is mediated by the amino-terminal ligand binding domain of the receptor, which forms a large hydrophobic cavity able to accommodate the protruding loop of ephrin-A1 (G–H loop) [Himanen et al., 2012]. The binding between receptor and ligand is due to van der Waals contacts between two predominantly hydrophobic surfaces, and two salt bridges between EphA2 Arg 103 and ephrin-A1 Glu 119 are necessary to reinforce [Fig. 39 A].

Binding interfaces in EphA2–ephrin-A1 complex are large, but peptides of moderate size (12 amino acids), as well as small molecules, exemplified by salicylic acid derivatives are able to occupy the same EphA2 receptor cavity as the G–H loop of the physiological ephrin ligands [Noberini et al., 2011].

Through modeling analysis performed by the group of researchers in Pharmaceutical Chemistry of the University of Parma, an hypothesis about the binding mode of LCA on EphA2 receptor was generated. The compound inserts its cyclopenta[a]perhydrophenanthrene scaffold the same space as the ephrin-A1 G–H loop placed into the hydrophobic Eph receptor channel. The carboxylic group, emerging from position -17 of the lithocholic acid core, forms a salt bridge with Arg 103, mimicking the interaction with Glu 119 from ephrin-A1, while the other opposite end, represented by the 3-hydroxy group interacts with Arg 159 of EphA2, which is usually engaged in a hydrogen bond with Asp 86 of ephrin-A1 [Fig. 39 B]. Docking analysis revealed that the interaction with Arg 159 is important for the initial stage of binding leading the steroid ring of LCA inside the hydrophobic channel of EphA2, but it is not essential for the activity, because the interaction was lost quickly.

Moreover computational results pointed out that while substituents in -7 or -12 are not tolerated for the activity, probably because of the bulky interaction with the aromatic ring of receptorial Phe 156, the carboxylic group in position -17 is important for the binding to EphA2 receptor.

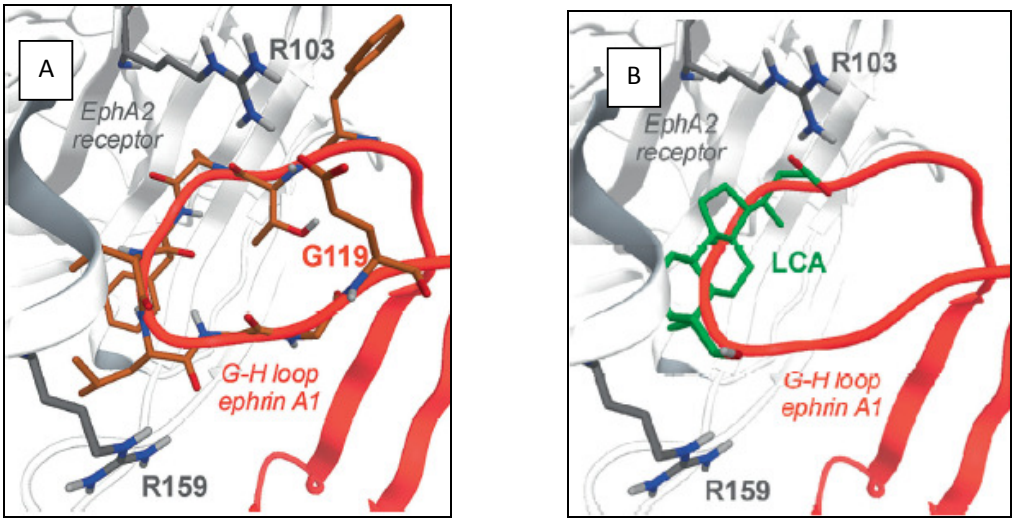


Figure 38: A. X-ray three-dimensional structure of the EphA2–ephrin-A1 complex. The amino-terminal domain of EphA2 forms a large hydrophobic cavity able to accommodate a protruding domain of ephrin-A1 (G-H loop). In evidence the salt bridge between R 103 (EphA2 receptor) and G 119 (ephrin-A1 ligand) [Himanen et al., 2009]. **B. Docking of LCA within EphA2 receptor.** LCA inserts its cyclopenta[a]perhydrophenanthrene scaffold into the hydrophobic Eph receptor channel occupying the space of ephrin-A1 G-H loop domain. The lateral acid chain of LCA forms a salt bridge with R 103, mimicking the interaction undertaken by ephrin-A1 G 119. LCA 3-hydroxy group interacts with R 159, usually involved in a hydrogen bond with Asp 86 of ephrin-A1 [Tognolini et al., 2012].

Considering these evaluations, LCA structure was used as a model to design and characterize new EphA2 ligands in order to obtain compounds with better pharmacodynamic features. Based on the computational results above 17 derivatives of LCA were chosen and tested for their ability to interact with EphA2–ephrin-A1 binding.

As we expected, modulation of the cyclopenta[a]perhydrophenanthrene group was detrimental for the activity (CA, DCA, CDCA), but also modifications in the lateral carboxylic group led to a loss of activity.

Interestingly, three compounds emerged from the new screening between LCA derivatives: isolithocholic acid, with an inversion of -3 hydroxy group from α (as in LCA) to β, cholanic acid, where -3 hydroxy group is absent and glycolithocholic acid, where the lateral carboxyl was derivatized with a glycine.

First of all this result confirmed that the -3 hydroxy group was not essential for the binding and that the inversion or the completely absence of it could improve the activity. On the other hand the higher activity of glycolithocholic acid, compared to LCA, suggested that the lateral carboxylic group is important for the activity and cannot be modified but could be derivatized, for example with a glycine as in this case.

Among the three compounds cholanic acid showed to be more affine than LCA (and all the other of this series) on ligand binding domain of EphA2, with concentrations in the low micromolar range.

Cholanic acid competitively and reversibly displaced biotinylated ephrin-A1 from EphA2 receptor: saturation curves obtained with increasing concentrations of the compound produced a Schild plot indicating competitive antagonism, and SPR analysis further confirmed the binding to EphA2 receptor. Moreover, cholanic acid was able to dose-dependently inhibit EphA2 phosphorylation induced by ephrin-A1 in cellular assays and the retraction of PC3 cells stimulated by ephrin-A1 ligand. On the other hand, the compound showed lower potency values in the inhibition of ephrin-B1-dependent phosphorylation of EphB4, according to the results obtained in the *in vitro* displacement assay. If cholanic acid had no effect on EphA2 kinase domain, isolithocholic acid was able to significatively inhibit it in the enzymatic assay at the concentration of 100 μM.

The binding affinity of cholanic acid and its ability to block EphA2 activity in cellular assays, supports the hypotesis that the cholanic acid structure can be used as a new scaffold to design an improved generation of EphA2 ligands. On the other hand, cholanic acid suffers from high lipophilicity determining unfavorable physicochemical properties. Further investigations should evaluate the development of cholanic acid derivatives endowed with higher solubility.

The considerations above about glycolithocholic acid led us to the second series of LCA derivatives. The inhibitory activity of the glycine derivative on EphA2–ephrin-A1 binding suggested that the carboxylic group was important for the activity but also that the derivatization of the acidic chain with amino acid including glycine could still produce compounds able to interact with Arg 103 of the receptor.

For these reasons, our collaborators (Dr. Alessio Lodola, Dr. Matteo Incerti and Dr Simonetta Russo) kindly projected and synthesized a new extended set of α-amino-acid derivatives of LCA which were evaluated for their ability to disrupt the binding of ephrin-A1 to the EphA2 receptor.

In this new series, small hydrophilic groups were tolerated, but bulkier substituents produced compounds completely inactive. On the other hand both small or large hydrophobic derivatives showed an increased potency. The best activity values were exhibited by aromatic and lipophilic substituents, including the phenylalanine and tryptophan derivatives, especially the L-Trp conjugate showing a pIC_{50} value of 5.69.

Computational studies highlighted the presence of an accessory hydrophobic site in the ligand-binding channel of the EphA2 receptor where the α-amino acidic chain of the compounds can be accommodated. This could explain the lack of activity for the relatively more polar derivatives, while a significative increase in pIC_{50} values was observed for the aromatic derivatives characterized by a phenylalanine or a tryptophan moiety. Hydrophobic interactions between L-Trp LCA and EphA2 receptor resulted to be important for the activity of this derivative. Moreover, the indole ring of L-Trp LCA tightly interacts with Phe 108, a conserved residue responsible for the recognition of one of the two aromatic residues (Phe 111) of ephrin ligands [Fig. 40].

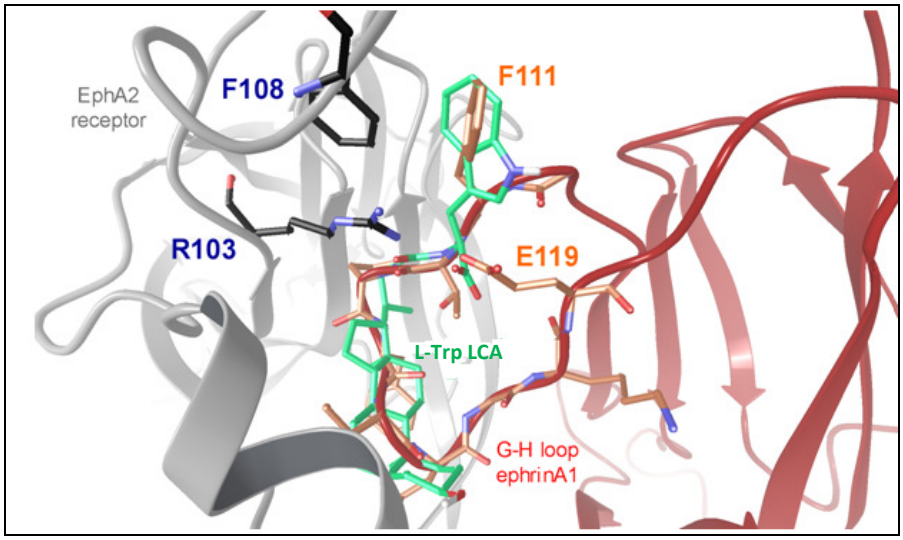


Figure 40: L-Trp LCA docking simulation. L-TrpLCA is shown in green. The structure of ephrin-A1 ligand is also displayed (red), as it appears in the X-ray structure of the EphA2–ephrin-A1 complex [Himanen et al., 2009].

L-Trp LCA was the best compound of the series in the inhibition of EphA2–ephrin-A1 binding. It was able to dose-dependently displace ephrin-A1 from immobilized EphA2 in low micromolar range and the binding was reversible. The saturation curves suggested a competitive behavior also if the Schild plot revealed a slope of 2.45, indicating that probably the mechanism of action is different than the other LCA derivatives: maybe due to the particular conformation of the compound the binding interaction between L-Trp-LCA and the receptor is not 1:1 as the other derivatives but 1:2, so one molecule

for two receptors. Further studies to elucidate the mechanism of binding should be performed. L-Trp LCA was able to inhibit the EphA2 phosphorylation induced by ephrin-A1 and to block the PC3 cells EphA2-mediated retraction induced by ephrin-A1. Finally it inhibited the tube formation of HUVECs, exhibiting good antiangiogenic features.

Despite the good activity showed by L-Trp LCA, the compound has untoward physicochemical properties (i.e. low solubility), and potential unknown activity also in other pathways which renders clueless the effect expectations *in vivo*. However, L-Trp conjugate symbolizes one of the most potent non-peptide antagonist of EphA2 receptor: for instance, 2,5-dimethyl-pirrolyl derivative showed good activity in preventing Eph-ephrin binding in the low micromolar range, but it was able to block EphA2 activity only at very high concentrations [Noberini et al., 2008]. Certainly, through the synthetic approach L-Trp conjugate could be a starting point for the design of better antagonists of Eph receptors with an improved drug profile.

2. Polyphenols

Through an initial ELISA binding assay screening between a variety of substances, nine phytocomplexes from edible plants showed the ability to interact with EphA2–ephrin-A1 system. Dose-response relationships both in binding and functional assays were obtained and the active concentrations in the binding study were comparable with the working concentrations in the functional assay. Moreover, the interaction between EphA2 and the exctracts in displacement studies was reversible and the exctracts showed in cellular assays performed on PC3 cells to be completely inactive on EGFR, suggestinfg the specificity of the action. The inhibition of EphA2 phosphorylation induced by ephrin-A1 was obtained at not cytotoxic concentrations: none of the extracts modified cell viability up to 40 µg/ml.

Interestingly, a moderate correlation between total polyphenolic content and both binding and phosphorylation results has been found [Fig. 41], while correlation with flavonoid and procyanidin content was neglegible [Tabel 4].

	Total polyphenols	Total flavonoids (g/100 g)	Total procyanidins
Azadirachta indica	53.81 ± 0.14	5.86 ± 0.07	1.718 ± 0.011
Ginkgo biloba	61.1 ± 0.10	25.13 ± 0.09	0.678 ± 0.010
Terminalia chebula	88.02 ± 0.21	5.64 ± 0.06	0.069 ± 0.004
Terminalia bellerica	59.84 ± 0.22	4.87 ± 0.07	0.073 ± 0.004
A. uva-ursi	68.36 ± 0.13	4.27 ± 0.05	0.171 ± 0.009
Punica granatum	87.08 ± 0.16	27.32 ± 0.04	0.004 ± 0.001
Lagerstroemia speciosa	56.88 ± 0.12	17.13 ± 0.04	0.268 ± 0.003
Phyllanthus emblica	47.08 ± 0.21	1.48 ± 0.02	0.108 ± 0.008
Embelia ribes	9.79 ± 0.05	1.7 ± 0.05	0.449 ± 0.012

Tabel 4: Phytochemical profile of most active plants. Polyphenols, flavonoids and procyanidin content ±SEM.

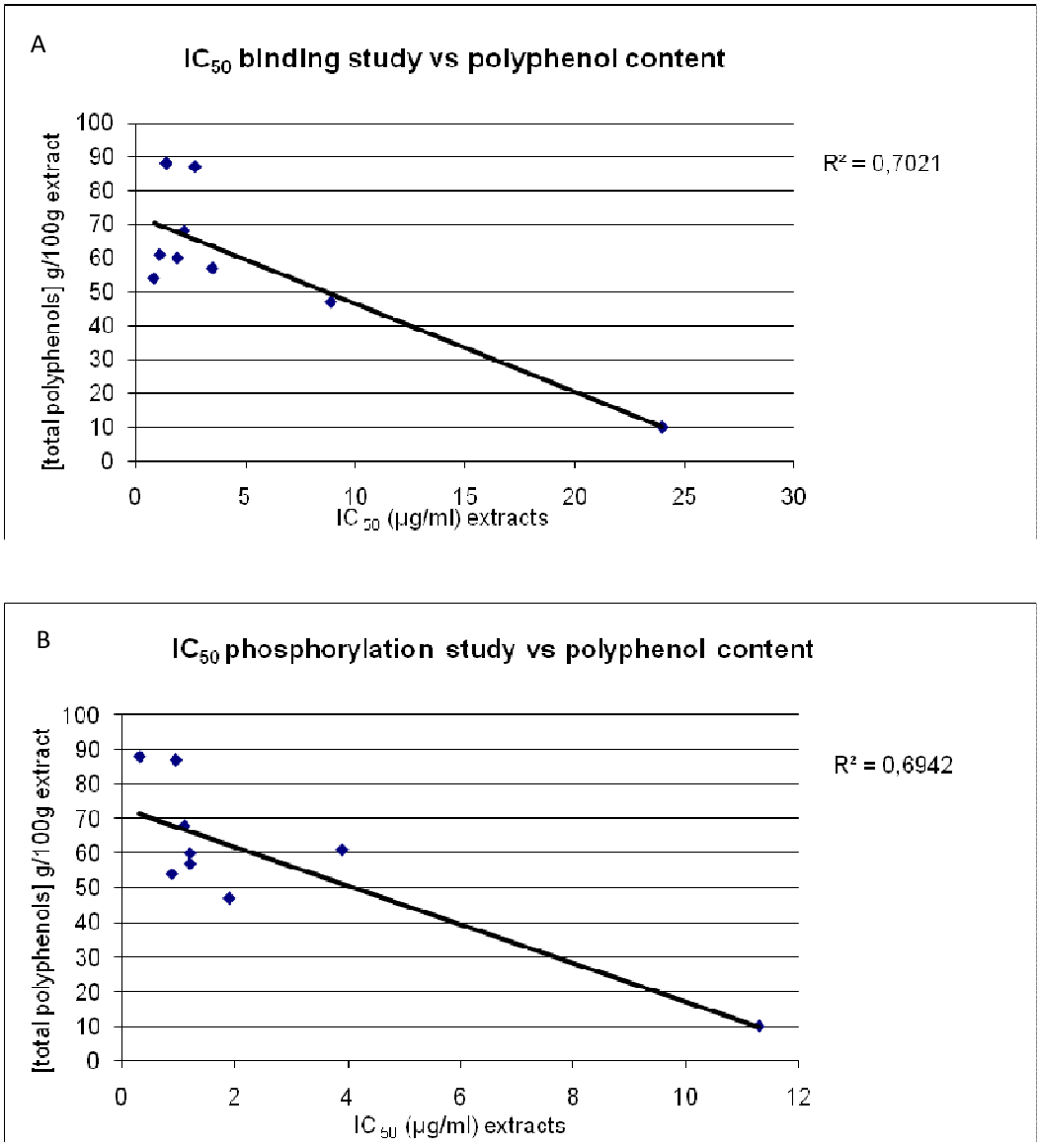


Figure 41: Correlation between total polyphenol content in extracts and IC_{50} values. A moderate correlation between total polyphenol content and IC_{50} values obtained both in the binding (**A**) and functional studies (**B**) was detected. The correlation with flavonoid and procyanidin content was negligible both in the binding (R^2 = 0,0377; R^2 = 0,0073 respectively) and functional study (R^2 = 0,0352; R^2 = 0,006 respectively) (data not shown).

As shown in Table 4 some of the most active extracts, including *A. indica, G. biloba* had an high content in procyanidins, but their action was similar to those provided by

phytocomplexes with a lower content as for *T. chebula, T. bellerica* or even almost without this class of polyphenols, as shown by *P. granatum*. On the other hand, *E. ribes* extract, exhibited the lowest IC_{50} both in binding and phospohorylation assays, but it contains a considerable amount of procyanidins. Probably, different polyphenolic substances could be potentially involved in the perturbation of Eph-ephrin system and may warrant further distinct phytochemical evaluation of some extracts.

Most of the active plants (*T. chebula, T. bellerica, P. emblica, E. ribes* and *A. indica*) are used in the ayurvedic medicine for a number of usages, including cancer prevention [Aggarwal et al., 2006; Balachandran et al., 2005] and the exctracts of these plants are obtained for simple decotion, the dietary form by which these plants are ingested. Three of them (*P. emblica, T. chebula* and *T. bellerica*) widely used in this alternative medicine are known to reduce metastasis *in vivo* and increase survival in cancer-affected mice [Menon et al., 1997; Baliga et al., 2010]. Since Eph-ephrin system is involved cell migration and angiogenesis, further investigations about the role played by these phytocomplexes could be useful [Brantely et al., 2002; Fang et al., 2005].

Also pomegranate fruit and ginkgo leaves, which extracts were active in our studies, are frequently used as functional food ingredients for their ability in supporting health benefit and antiangiogenic properties. Different studies reported the ability of *A. indica* and *G. biloba* to reduce tumor growth in different kind of cancers and this effect could be associated to their antiangiogenic properties and their involvement in the regulation of cell proliferation and differentiation, but the real mechanism of action of this extracts was not available [Vimal et al., 1992; Lansky et al., 2007; De Feudis et al., 2003; Kumar et al., 2005].

No correlation between antitumoral activity and both *L. speciosa* and *Arctostaphylos uva-ursi* have been found, also if our studies showed their ability in the modulation of Eph-ephrin system.

Interestingly, the food intake of these phytocomplexes is associated with a decrease of cancer risk. All the nine extracts showing activity in our assays are known to contain polymeric polyphenols and tannins, which often are not-specific inhibitors of enzymatic system and proteins. It has been shown that tea polyphenols derived from black and green tea could suppress the biological activities of Eph receptors in cells also if a potential additional mechanism of inhibition could be involved in the interference with ephrin binding [Noberini et al., 2012].

Neverthless, since Eph-ephrin system is involved in intestine self renewal regulating cell migration patterns on the crypt-villus axis [Kosinski et al., 2007; Moore et al., 2006]

and may act as potential tumor suppressors if properly modulated, the perturbation by food-related phytocomplexes may suggest intriguing pathophysiological implications [Tognolini et al., 2012; Cortina et al., 2007; Batle et al., 2002].

For these reasons, considering the role played by Eph-ephrin system in many diseases including cancer and pathological angiogenesis, these extracts could be evaluated not only for further investigations about the role of Eph-ephrin system in cancer but also for the pathophysiological implication in intestine homeostasis.

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