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IDENTIFICATION AND PHARMACOLOGICAL CHARACTERIZATION OF NOVEL EPH/EPHRIN ANTAGONISTS

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INTRODUCTION

The EPH/EPHRIN SYSTEM

Family and structure

Eph receptors (Erythropoietin-Producing Hepatocellular carcinoma) are the largest family of tyrosine kinases receptor (RTKs) and together with their ligands, the ephrins, are important mediators of cell–cell communication, by regulating cell attachment, shape, and mobility in neuronal and endothelial cells.

The Eph receptors are subdivided in two classes A and B, based on sequence homology and binding affinity for the ephrins, which are membrane-anchored proteins classified in A and B. Ephrins-A are anchored to the cellular membrane by a glycosyl-phosphatidyl-inositol (GPI)-linkage, whereas ephrins-B possess a single transmembrane domain and a short cytoplasmatic domain that contains a cytosolic PDZ-binding motif [Pasquale 1997].

In humans, the EphA class includes nine members (EphA1–A8, EphA10) which interact with five ephrins-A (ephrin-A1-A5), while the EphB class consists of five members (EphB1–B4, EphB6) and they bind three ephrins-B (ephrin-B1-B3). However, some promiscuous binding between classes have been noted (Fig. 1), for instance, EphB2 can bind ephrin-A5 beside all ephrins-B, as well as EphA4 can interact with several ephrin-Bs [Himanen et al. 2004; Kullander and Klein 2002].

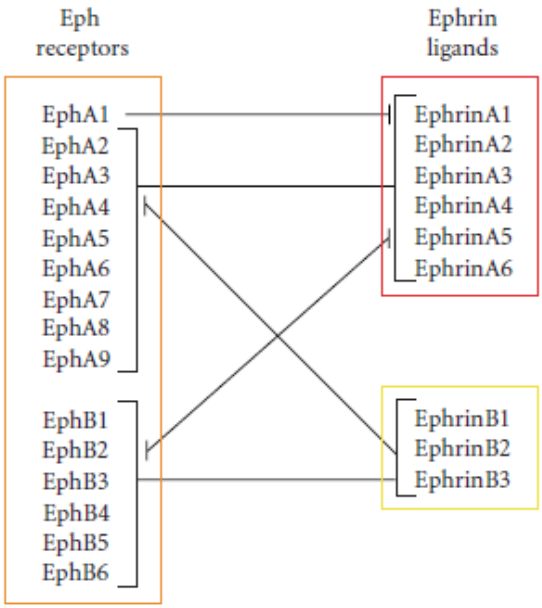


Figure 1: Major interactions of Eph receptors and ephrin ligands (Mosch et al. 2010).

EphA and EphB receptors show a similar protein structure by sharing (Fig. 2):

-an extracellular region including the ligand binding domain (LBD), situated in the N-terminal domain, a cysteine rich-domain (encompassing a sushi domain and an epidermal growth factor (EGF)–like motif), and two fibronectin-type III repeats [Mercurio and Leone 2016];

-an intracellular region containing a juxtamembrane domain, a tyrosine kinase domain, a sterile alpha motif (SAM), and a PDZ-(Postsynaptic density, Discs large, Zonula occluden-1) binding motif [Hirai et al. 1987].

EphA10 and EphB6 are two exceptions since they lack of the kinase activity due to altered sequence of the conserved regions within the kinase domain [Mendrola et al. 2013]

In the ligand binding domain, high affinity binding site is found together with two low-affinity sites. The former mediates the Eph-ephrin interaction among adjacent cells whereas the latter are important for the formation and stabilization of clustered Eph-ephrin complexes [Pasquale 2008].

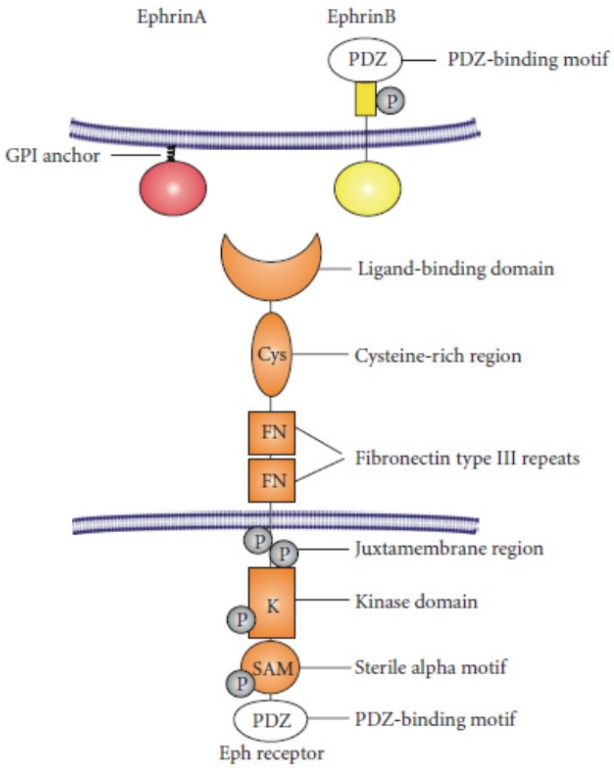


Figure 2: Structure of Eph receptors and ephrin ligands (Mosch et al. 2010).

The Eph-ephrin signalling

The Eph/ephrins system shows unique properties in the activation and signalling.

A direct cell-cell contact is necessary to have the interaction between receptor and ligand, when Ephs and ephrins are located on opposite cells (trans-configuration).

The trans Eph/ephrin interaction triggers a bidirectional signalling (Fig.3) both in Eph-bearing cell, called forward signalling, and in ephrin-expressing cell, known as reverse signalling [Pasquale 2008].

The physical contact between Eph and ephrin leads to the clustering of ephrin-linked Ephs, to the trans-phosphorylation of intracellular tyrosine residues of the Eph receptor and finally to the activation of different downstream signal transduction cascades [Mosch et al. 2010].

In addition to the "*trans* configuration", Eph receptors and ephrin ligands can also be expressed on the same cells, *cis*-configuration (Fig 3). The *cis* binding would attenuate the formation of ephrin-linked Eph clusters in *trans* and thus the signalling [Pitulescu e Adams 2010].

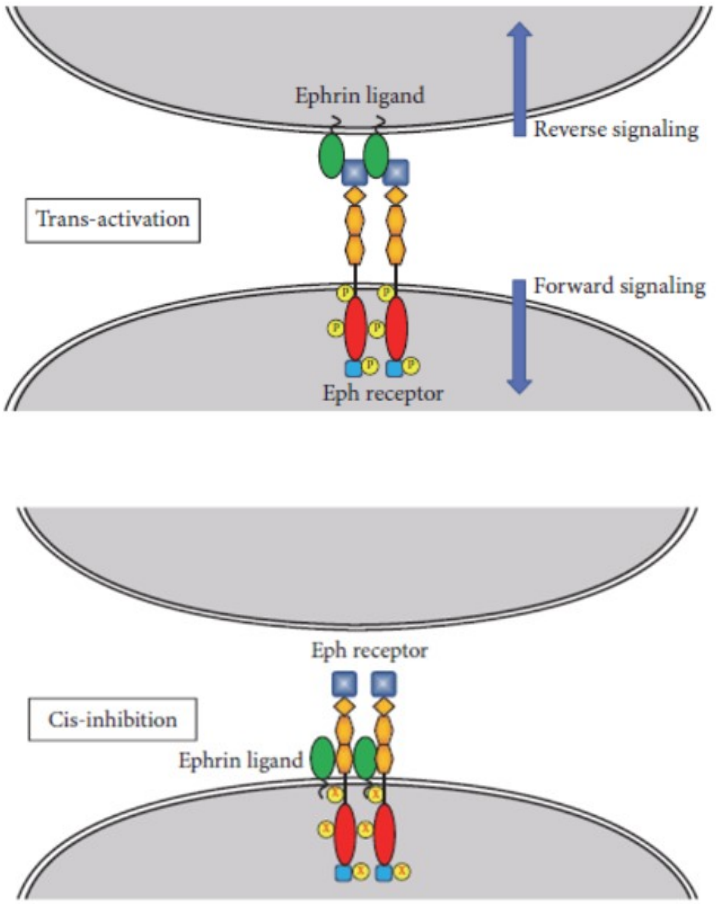


Figure 3: Bidirectional signalling activated by Eph/ephrin system [Kou and Kandpal 2018].

Once Eph receptors are phosphorylated, various effector proteins are recruited, including
- tyrosine kinase adaptor protein, containing Src homology 2 and 3 (SH2 and SH3) domains,
- kinases and phosphatases,
-guanine-nucleotide Exchange factors (GEF) as Vav2 and Vav3, ephexins,
- Rho GTPase-activating protein as α2-chimerin, involved in the remodelling of the actin cytoskeleton and cell adhesion

These effector proteins can act either as adaptors for enzymes located downstream in a signalling pathway or they can have direct enzymatic activity.

Moreover, some signalling connections are exclusive for a specific class of Eph receptors, as EphA receptors and the Rho exchange factor Ephexin or EphB receptors and the exchange factors Intersectin and Kalirin. [Kania e Klein 2016]

Although cell-cell contact is necessary for the Eph-ephrin interaction, it has been demonstrated that ephrins-A can be released from the cell surface by metallo-protease and activate EphA receptors in a paracrine manner, without juxtacrine interactions [Wykosky et al. 2008].

Beside the forward signalling, the reverse signalling elicited by ephrins-B, recruits Src kinases which phosphorylate tyrosine residues in the cytoplasmic domain, allowing the recruitment of adapter proteins containing either the PDZ domain or the SH2 domain and then the activation of cellular signalling pathways. For example, the recruitment of the Grb4 (SH2-adapter proteins) induces axon pruning [Lin et al. 1999][Xu and Henkemeyer 2009].

By contrast, ephrins-A ligands, as they lack the cytoplasmic domain, need to associate with transmembrane molecules to trigger cellular responses. Ephrins-A can interact with p75NTR neurotrophin receptor, with tropomyosin receptor TrkB or with Ret tyrosine kinase receptor. Cellular attraction takes place if ephrin-A couples with Ret, whereas the interplay with p75NTR, involving the Src family kinase Fyn, mediates cell repulsion [Kania and Klein 2016].

Generally, the shutdown of the signal occurs when the Eph-ephrin interaction is interrupted and it can happen with different mechanisms. The Eph-ephrin signalling is terminated through recruitment of phosphatases, which dephosphorylate the tyrosine in kinase domain, adaptor proteins involved in endocytosis and degradation of receptor, and through proteases which cleave the Eph receptor and ephrin in the extracellular (as for ADAM family metalloproteases), intracellular (γ-secretase) or in the transmembrane regions.

The activation of Eph-ephrin signalling regulates different cellular functions, as motility, morphology, cell repulsion and adhesion by rearranging the cytoskeleton and by influencing the activities of integrins and intercellular adhesion molecules [Egea and Klein 2007; Himanen et al.

2007]. Also, effects on cell proliferation, survival, differentiation, and secretion have been related to this system.

Crosstalk with other signalling systems

A cross-talk between Eph receptors/ephrins and other signalling pathways is well known, in fact, even more studies provide evidences of interactions between Eph/ephrin and other tyrosine kinase receptor (RTK), adhesion molecules and other different cell surface receptors.

Fukay et al. observed the complex formation between EphA4 and the fibroblast growth factor receptor 1 (FGFR1) in the cells, that enhances cell proliferation and migration induced by activated FGFR1 [Fukai et al. 2008].

Larsen et al. described, instead, a cooperation between epidermal growth factor receptor (EGFR) and EphA2. Activated EGFR would increase the expression of EphA2 in human cancer cell and it would promote cell motility and proliferation [Larsen et al. 2007].

Moreover, EphA2 has been found to enhances the Epithelial-mesenchymal transition via activation of Wnt/β-catenin signalling in gastric cancer cell. Recent evidences suggest as EphA2 would facilitate the translocation of β-catenin to the nucleus, by binding proteins involved in the β-catenin degradation complex [Peng et al. 2018].

The Eph-ephrin system in physiology

Eph/ephrin signalling has been shown to elicit several relevant cellular responses in embryogenesis and for homeostasis adult tissues. During embryogenesis this system is highly expressed, and it plays key roles in tissue compartmentalization and bone development, as well in the formation of cardiovascular and nervous system.

In embryonic tissues the Eph receptor distribution is complementary to the ephrin one and this allows the compartmentalization of adjacent cell populations through repulsive and adhesive processes generated after Eph-ephrin interaction [Dodelet and Pasquale 2000; Gale and Yancopoulos 1997].

Physiological functions regulated by the Eph-ephrin system are angiogenesis, bone and intestinal homeostasis, platelet aggregation, immune system function, inflammation and glucose balance. Moreover, Eph receptors and ephrin ligand have been found expressed in adult stem cell as neural, intestinal and hematopoietic stem cell, where they exert a control function of adult stem cell niche [Kania and Klein 2016].

The Eph-ephrin system in Central nervous system

Contrary to the major expression during the development of nervous system, Eph receptors and ephrin ligands are mainly found in the adult brain in areas with increased remodelling of neuronal circuits or in regions important for memory and learning process [Yamaguchi and Pasquale 2004] where they modulate the number and size of synapsis.

Moreover, the expression pattern of Eph receptors and ligands differs both between various brain regions and in the synapses of the same neuron [Dalva 2007]. To mention an example, EphB receptors and EphA4 are over-expressed in the hippocampus but EphB presents a post-synaptic localization whereas EphA4 owns pre and post-synaptic positioning [Tremblay et al. 2007].

Neural progenitor cell proliferation, neuroblast migration, neuron survival, and neuronal plasticity rely on Eph-ephrin signalling in the central nervous system. Conover et al. demonstrated as ephrin-activated EphB1, EphB2, EphB3, EphA4 induce migration of neuroblasts in the subventricular zone of the lateral ventricles in the adult brain [Conover et al. 2000] Holmberg et al., instead, discovered that the EphA7-induced ephrin-A2 reverse signalling negatively affects neural progenitor cell proliferation in mice and ephrin-A2 knock-out in the neural stem cell niche cells proliferate more and shorten their cell cycle. The author also proposed that ephrin-A2 and EphA7 would control the number of cells generated in the adult brain, through a feedback system between cells of different maturational states in the stem cell niche [Holmberg et al. 2005].

This means that the activation of bidirectional signalling would regulate the amount of proliferating neural progenitor cells in order to maintain the right number of cells in the brain.

The Eph-ephrin system in blood and lymphatic vessels development

Vasculogenesis and lymphangiogenesis are the development of new blood and lymphatic vessels respectively. These processes are highly influenced by the ephrin-B2 and EphB4 signalling, in fact, silencing of these proteins in mice determined severe defects in growth and remodelling of blood vessels [Wang et al. 1998]. These two proteins show a different pattern of expression: ephrin-B2 is located on the surface of endothelial cells of arteries whereas EphB4 is found on veins ones [Kania e Klein 2016].

As example, dorsal aorta and cardinal vein formation are two events which require the EphB4-ephrin-B2 signalling because it leads to the segregation of precursor venous endothelial cells, expressing ephrin-B2, from progenitor of arterial endothelial cells, expressing EphB4 [Lindskog et al. 2014].

Ephrin-B2-EphB4 signalling manages also the sprouting angiogenesis. As observed in the postnatal mouse retina, the activation and the internalization of $VEGFR_2$ (vascular endothelial growth factors receptor) by $VEGF_{165}$ induce cell sprouting, motility and proliferation of endothelial cells. Ephrin-B2 through Dab2 (clathrin-associated protein disabled 2) and PAR-3 (cell polarity regulator) facilitates the clathrin-dependent $VEGFR_2$ internalization and thus its downstream signalling. The positive regulation exerted by ephrin-B2 was demonstrated to rely on its PDZ interactions and it might be mediated by Src family kinases [Wang et al. 2010; Nakayama et al. 2013]. Ephrin-B2 PDZ-signalling-deficient mice, in fact, showed decreased vascularization, in term of vessel branching and number of vascular sprouts [Sawamiphak et al. 2010].

The role of ephrin-B2 in lymphangiogenesis was also investigated and it regulates the growth of lymphatic endothelium [Wang et al. 2010] in association with VEGF-C induced $VEGFR_3$ signalling. The activation of forward and reverse signalling generated after ephrin-B2-EphB4 interaction induces the internalization and promote the signalling of activated $VEGFR_3$ in lymphatic endothelial cells. As proof of that, mouse Ephrin-B2 knockout endothelial cell showed a compromised VEGF-C-induced $VEGFR_3$ endocytosis and a reduced activation of $VEGFR_3$ and of small GTPase Rac1 (a regulator of cell motility and protrusion formation).

However, in absence of VEGF-C, ephrin-B2 allowed only the $VEGFR_3$ internalization but not an appreciable activation of the receptor. This suggests that ephrin-B2 plays an important role for the $VEGFR_3$ signalling but it cannot replace the physiological ligand VEGF-C [Wang et al. 2010].

The Eph/ephrin signalling in cancer

In the last decades, an increasing number of studies have investigated the role of the Eph-ephrin system in cancer, even if it is not completely elucidated yet. Different expression and biological effects have been found for these proteins, depending on tissue, cellular context and pathway involved.

In some tumors, the overexpression of Eph receptors and the simultaneous downregulation of their ephrin ligands have been associated with high clinical stage tumors and with a poor prognosis. In other instances, tumor growth and increased metastasis formation have been related to the downregulation of Eph receptors, suggesting a tumor-suppressor activity for these proteins in this latter case. EphA2, EphB4, EphA3 are examples of receptors upregulated in glioma, ovarian and colorectal cancer [Li et al. 2010; Kumar et al. 2007; Xi e Zhao 2011], whereas EphA1, EphA7 and EphB6 are downregulated in colorectal, prostate and melanoma cancer cells respectively [Hafner et al. 2003; Guan et al. 2009; Herath et al. 2009].

The pro-oncogenic effect of Ephs is thought to be due to changes in cell adhesion, motility and cytoskeleton organization which would facilitate dissemination and invasion of tumor cells. For instance, in PC3 prostate cancer cells ephrinA1-dependent EphA2 signalling decreases the activation of focal adhesion kinase (FAK) by recruiting the protein tyrosine phosphatase SHP2, which results in the reduction of migration and integrin-mediated adhesion through conversion of integrins in inactive conformation [Miao et al. 2000]. However, in the absence of ligand, Akt which is hyper-activated in tumor cells (due to growth factor receptor activation or loss of PTEN) phosphorylates EphA2 on serine residue, S897 and this promotes cell polarization and cell migration. In this regard, Miao et al. attributed a pro-oncogenic and tumor-suppressive role to EphA2: the ligand-depend EphA2 signalling would inhibit the migration of prostate cancer cells, on the contrary, the ligand-independent EphA2 signalling would promote it. This would also explain the simultaneously over-expression and downregulation of EphA2 and ephrin-A1 in prostate cancer [Miao et al. 2009].

In EphA3-expressing melanoma cells, instead, the ephrinA5-induced EphA3 signalling produces cell rounding, membrane blebbing and de-adhesion through activation of RhoA [Lawrenson et al. 2002].

Also, survival and proliferation of tumor cells have been related to Eph/ephrin signalling. Malignant T lymphocytes overexpress EphB6 and EphB3 and the activation of their forward signalling by ephrin-B1 and ephrin-B2 suppresses the Fas-mediated cell death. This pro-survival effect was correlated to Akt activation which in turn, prevents the FasR-initiated apoptotic response [Maddigan et al. 2011]. Anti-apoptotic activity was observed also in ephrin-A2 overexpressing hepatocellular carcinoma cells, which showed an increase survival due to the resistance to tumor necrosis factor-α-(TNF-α-) induced apoptosis [Feng et al. 2010]. The EphB2 forward signalling, instead, displayed to enhance proliferation in colon cancer by means of Abl/cyclin D1 activation [Genander et al. 2009]

Finally, the Eph-Ephrin system plays a main role in tumor angiogenesis, crucial process for the survival and maintenance of tumor. Without angiogenesis tumor size is restricted, due to lack of nutrients, growth factors, and oxygen.

Tumor angiogenesis starts with the release of proteins by tumor cells or by infiltrating immune cells. The released proteins are distinguishable in pro-angiogenic, as bFGF, aFGF, and VEGF, and in anti-angiogenic as α-Interferon, platelet factor-4, and thrombospondin-1. It is just the altered ratio between these factors with the prevalence of pro-angiogenic factors which sustains tumor angiogenesis [Ribatti et al. 2007; Hanahan and Folkman 1996].

Beside tumor angiogenesis, cancer cells can create vascular structures mimicking blood vessels, through a mechanism called vasculogenic mimicry, which allows the tumor perfusion with nutrients and oxygen.

An increasing body of studies confirmed the involvement of Eph receptors in these two mechanisms. Hess and colleagues observed the suppression of tubular network formation consequently to downregulation of EphA2 expression in uveal melanoma cells [Hess et al. 2001], as well as, a decreased microvascular density was reported by Brantley-Sieders after inoculation of mammary adenocarcinoma cells in EphA2-deficient mice [Brantley-Sieders et al. 2005]. Also ephrin-A1 acts as a proangiogenic factor as revealed from the reduced *in vitro* endothelial cell migration and tumor microvasculature density *in vivo*, after siRNA-mediated knockdown of ephrinA1[Tandon et al. 2011]. Beside the Eph/ephrin A family, also EphB receptors and ephrinB ligands have shown to prompt tumor angiogenesis. In a study carried out by Kumar and colleagues, EphB4 was positively correlated with tumor growth, tumor resistance and tumor vasculature in breast cancer cells [Kumar et al. 2007]. As concerns the involvement of ephrin-B2 in tumor angiogenesis, it has been explored for the last ten years. Abengozar observed a reduction in the number of blood and lymphatic vessels in xenografted mice after administration of ephrin-B2-blocking antibody [Abéngozar et al. 2012]. Few years before, Sawamiphak et al. had demonstrated that ephrin-B2 reverse signalling was a positive regulator of $VEGFR_2$ endocytosis and function, not only in physiological but also in pathological conditions [Sawamiphak et al. 2010].

Prostate cancer

Prostate cancer is one of the main causes of cancer mortality in american men. For several years the monitoring of PSA (Prostate Specific Antigen) serum levels has been used for screening and follow-up of the diseases. However, this marker possesses some limitations when it is used as prognostic marker as it does not allow to correctly distinguish between responding or not-responding patients to a given treatment or between indolent and aggressive form of cancers. [Romero Otero et al. 2014] Thus, the identification of new and more reliable markers is an important task for a better prognosis and in this scenario Eph receptors could turn out not only as useful markers during histological analysis of tumors but also as potential therapeutic targets. Overexpression of EphA2 and EphA5 have been observed in prostate cancer cells while weak or absent expression has been reported in normal cells [Walker-Daniels et al. 1999]. Additionally, the expression levels of the EphA2 receptor have been negatively associated with patient survival rate.

Taddei et al. demonstrated as EphA2-overexpressing prostate carcinoma cells initiate a series events, as regulation of an amoeboid-based invasive strategy and increase of stem cell markers, which enhance dissemination to bone and growth of metastasis [Taddei et al. 2011].

EphB4 is another receptor highly expressed in prostate cancer and it was linked to the development and progression of the tumor. It was observed a reduced viability, migration and invasion in tumor

cells where EphB4 was knockdown. The pro-oncogenic role of this receptor was demonstrated not only *in vitro* but also *in vivo*, in fact, knockdown of EphB4 resulted in a suppression of tumor growth [Xia et al. 2005].

Finally, in addition to overexpression, some Eph receptors are mutated or inactivated in prostate cancer cells and EphB2 is just an example. Huusko et al. identified eight mutation for EphB2 and six of them are localized in the intracellular region, especially on the kinase domain. It is plausible that EphB2 receptor plays a tumor suppressor role and the occurrence of these mutations would allow cancer progression and metastasis. To reinforce this hypothesis, cancer cells bearing wild-type EphB2 proliferated less and formed less colonies than tumor cell with EphB2 mutated. [Huusko et al. 2004].

Glioblastoma

Glioblastoma multiforme (GBM) is the most malignant primary brain tumor in adult, highly aggressive, with marked heterogeneity and resistance to conventional therapies, including surgical resection, radiotherapy and chemotherapy [Wen e Kesari 2008]. The median survival of patients is less than 15 months and only 10% of surviving patients do not develop recurrence after two years [Stupp et al. 2005]. Glioblastoma arises from glial cells as astrocytes and oligodendrocytes which normally serve supporting roles within the nervous system. Based on molecular featured and genetic alteration, glioblastoma has been classified in 4 groups (classical, mesenchymal, neural and proneural) [Verhaak et al. 2010].

The main causes of lethality of this tumor are:

-the diffuse infiltrative invasion of GBM cells throughout the brain,

-the presence of stem-like cancer cells which are responsible for recurrence after surgical resection of primary tumors,

- the robust angiogenesis.

Genomic studies highlighted that the great majority of GBMs exhibit genetic alterations in several signalling pathways as the P_{53}, the RB, and the RTK pathway [Parsons et al. 2008]. These mutations would enhance cell proliferation, survival and help tumor cells to escape from cell-cycle checkpoints, senescence, and apoptosis.

Altered expression of ephrins and Eph receptors have been also found in GBM and numerous studies have tried to elucidate if these proteins might promote or inhibit progression in GBM.

Recent findings highlighted as EphA2, EphA3 EphA7 are usually over-expressed in GBM and their expression was associated with poorer patient prognosis as revealed by comparing the overall and progression-free survival of patients with tumors positive or negative for EphA2. Li et al., observed

a reduced cell viability, invasion, and increased apoptosis in glioblastoma cells where endogenous EphA2 had been suppressed [Li et al. 2010]. Conversely, elevated expression of ephrin-A1 and ephrin-A5, the preferred ligands for EphA2 and EphA3, correlates with less-aggressive and more-differentiated phenotype of GBM [Binda et al. 2012; Liu et al. 2007; Wykosky et al. 2008; Day et al. 2013]. According to Li et al., Ephrin-A5 might act as a tumour suppressor, by enhancing c-Cbl binding to EGFR, thus promoting ubiquitination and degradation of the receptor [Li et al. 2009].

Moreover, recent studies have revealed high levels of EphA2 and EphA3 on tumor-initiating cell population in GBM, as demonstrated by the co-expression of CD133 and integrin α6 (GBM progenitor cell markers) with EphA3, as well as, Sox2 and Nestin (neuronal stem markers) with EphA2. These receptors are thought to keep tumor cells in an undifferentiated state, in fact, when EphA2 and EphA3 are silenced by small interfering RNA or downregulated with ephrin ligand stimulation, tumor-initiating cells miss their stem properties as self-renewal and clonal capacity, and become more sensible to traditional treatments [Day et al. 2013; Binda et al. 2012]

The mechanism through EphA2 and EphA3 maintain GBM cells in a stem-like state is probably through a negative regulation of MAPK/ERK1/2 pathway. Sustained ERK1/2 signalling was observed after knockdown of EphA3 in a human glioblastoma cell line, U251, leading to increased cell differentiation and reduced proliferation. Moreover, when EphA3 knockout U251 cells were subcutaneously injected into nonobese diabetic severe combined immunodeficiency mice, a dramatic reduction in tumor growth and an increased overall survival were observed compared to mice bearing tumors arising from EphA3 expressing U251 [Day et al. 2013]. Same results were obtained by Binda et al. after intracranial injection of EphA2 knockout tumor propagating cells. [Binda et al. 2012]

These studies suggest that a transient ERK1/2 activation is a positive signal for cells by promoting proliferation and survival, whereas a sustained activation becomes detrimental, leading to activation of differentiation and apoptosis signals.

In GBM EphA2 and EphA3 might act as switches, by regulating the duration of ERK activation, and subsequent ability of these cells to undergo differentiation [Day et al. 2014].

Colorectal cancer

Colorectal cancer is the fourth most deadly cancer in the world and the incidence of this disease is particularly high in riches countries due to adoption of inappropriate lifestyle and unhealthy Western diet [Brody 2015]

In a normal intestinal epithelium, several Eph receptors and ephrin ligands are expressed in gradient along the colon crypt axis and localized on the luminal surface where they control the correct cell

positioning along the crypt–villus axis and the proliferation of intestinal stem cells. [Batlle et al. 2002]

Many studies have reported a modified expression of Eph receptors in colorectal cancers, especially for EphA2, EphA1, EphB4 and EphB2. Overexpression of EphA2 has been found in the early stages of disease and it was positively correlated with high expression of stem cell marker. Moreover, high EphA2 expression was associated both with poor overall survival of patients and with an increased migratory and invasive capabilities of cancer cells. Down-regulation of EphA2 using RNAi or ephrin-A1 determined suppression of *in vitro* migration and invasion. [Dunne et al. 2016]

EphB4 and EphB2 showed an opposite expression in colon cancer cells. On the one hand, EphB4 is found on colon cancer cells and its expression levels relates with stage and grade of colon cancer. [Kumar et al. 2009]. On the other hand, the EphB2 expression lower with the tumor progression, firstly abundant on colon progenitor cells. [Batlle et al. 2005]

Finally, EphA1 was also proposed as prognostic marker due to the upregulation in colorectal cancer, especially during the early stages. However, EphA1 downregulation is common event in metastatic and in grade III colon cancer and it is associated with a lower survival [Herath et al. 2009].

Strategies to inhibit the Eph-ephrin signalling

The overexpression and the altered function of both Eph receptors and ephrin ligands make this system a potential interesting target in oncology.

To date, there are different strategies to target Eph receptors and ephrins (Fig.4):

- intracellular block of tyrosine kinases activation,
- extracellular inhibition of the receptor-ligand interaction,
- downregulation of Eph receptors and ephrins expression by using antisense oligonucleotides or small interference RNAs. These molecules eliminate all activities of their targets, but they lack of an efficient *in vivo* delivery which hampers their utilization.

The second strategy, unlike the first one, allows to block not only the forward but also the reverse signalling.

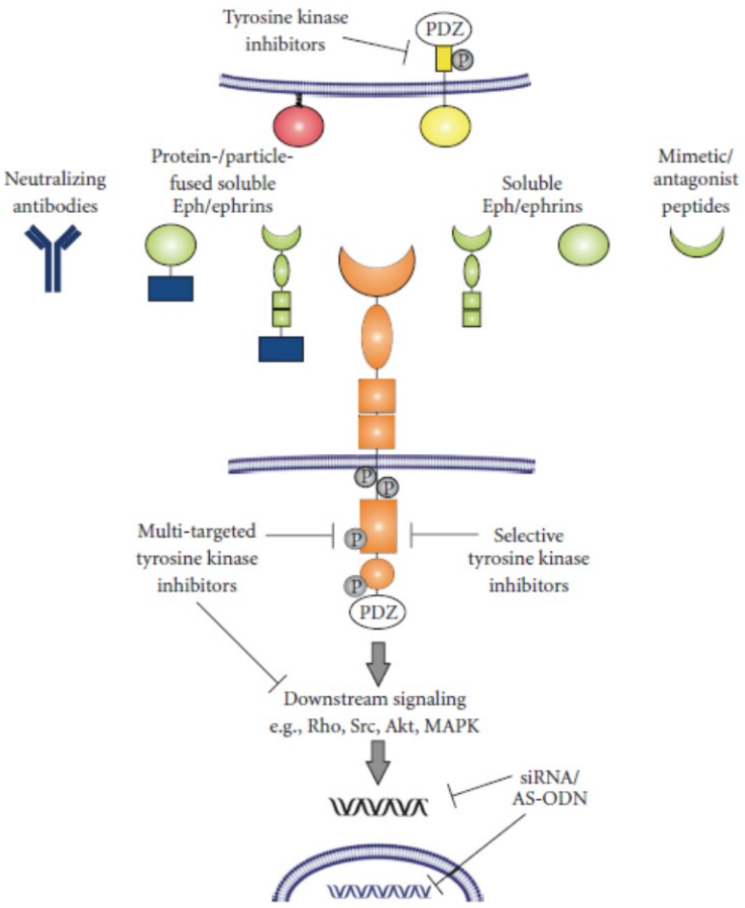


Figure 4: Strategies for intracellular and extracellular inhibition of the Eph-ephrin signalling [Mosch et al. 2010]

Intracellularly inhibition of tyrosine kinases activation

Kinase inhibitors bind with nanomolar affinity to the ATP-binding pocket of Eph kinase domain, by blocking the phosphorylation and thus the activation of the receptor. However, this class of compounds is featured by some pharmacological issues, as poor selectivity, because they usually target multiple kinases, and poor access to their intracellular targets. An example is Dasatinib. Initially, designed to target Bcr-Abl and Src family kinases, it has shown to have EphA2 kinase as an additional target. [Huang et al. 2014].

JI-101 is another oral multi-kinase inhibitor targeting multiple angiogenic pathways such as VEGFR2, platelet derived growth factor receptor β (PDGFR-β) and EphB4. JI-101 was studied in combination with Everolimus in a phase I trial and it led to the stabilization of disease for 4 months in patients with advanced solid tumors [Werner et al. 2015].

Some efforts have been done to develop inhibitors selective for a single tyrosine kinases. Just to mention, the 3,5-disubstituted anilinopyrimidines specific for EphB4 receptor [Bardelle et al. 2008], or recently synthetized quinazoline derivates which are irreversible inhibitors of EphB3 kinase [Kung

et al.2016]. However pharmacological *in vivo* studies by proving their activity and their application in cancer therapy have not been done yet.

The first evidence of in vivo efficacy was obtained with NVP-BHG712, a potent and selective kinase inhibitor of EphB4. The compound has shown to inhibit the VEGF driven angiogenesis in mice, without interference with VEGFR2 receptor and to increase the levels of paclitaxel in tumors but not in plasma when co-administered with paclitaxel. This latter association could turned out useful especially for the treatment of paclitaxel resistant tumors. [Kathawala et al. 2015; Martiny-Baron et al. 2010]

Finally, GLPG1790 is a noteworthy small molecule targeting the intracellular kinase domain of various Eph receptors which has showed anti-tumor activity *in vivo*. GLPG1790 was able to reduce tumor growth in orthotopic models of glioblastoma, to decrease the expression of mesenchymal stem markers (CD44, Sox2, nestin, CD90, and CD105) and to increase differentiation markers in glioma initiating cells [Gravina et al. 2019].

Extracellularly avoidance of the receptor-ligand interaction

As noted above, another strategy to interfere with the Eph-ephrin signalling is to prevent the interaction between receptor and ligand. Protein-protein interaction inhibitors hinder the binding of ephrin ligands to the extracellular ligand domain of Eph receptors. Peptides, antibodies, soluble forms of Eph or ephrin, and small molecules belong to this class.

Peptides: A series of 12 aminoacid-long peptides have been identified and they have shown to selectivity bind the ephrin-binding pocket domain of different EphB receptors, including EphB1, EphB2, and EphB4. Among them, the TNYL-RAW peptide was able antagonize ephrin-B2 binding to the EphB4 with nanomolar affinity comparable with that of the natural ligand [Koolpe et al. 2005]. The cyclic form of this peptide was conjugated with doxorubicin-loaded nanosphere and it showed a complete tumor regression, due to the combination of selectively delivery chemotherapic agent to EphB4-positive cancer cells and of photothermal heating damaging in mouse tumor xenografts [You et al. 2012]. Moreover, a recent work published by Bhathia et al. has observed as radiation combined with TNYL-RAW significantly improved the radiosensitivity and suppressed the tumor growth in orthotopic model of head and neck squamous cell carcinoma. The peptide modified the tumor immune microenvironment, by reducing the number of Treg lymphocytes and by shifting the polarization of macrophages from the pro-tumoral M_2 to the anti-tumoral M_1 phenotype [Bhatia et al. 2019].

YSA and SWL are, instead, two EphA2 agonist peptides and the former has been coupled with magnetic nanoparticles to remove EphA2-positive ovarian cancer cells from the peritonea of

experimental mice [Scarberry et al. 2010]. Moreover paclitaxel-conjugated YSA inhibited tumor growth more effectively than paclitaxel alone, when tested in PC3 xenografted nude mice [Wang et al. 2013]

Monoclonal antibodies recognize the extracellular domain of Eph or ephrin and they are useful tools to modulate the Eph-ephrin system, due to their high binding affinity, specificity and their long *in vivo* half-life.

Antibodies against EphA2, EphA3 and ephrin-A4 have provided promising results in some preclinical models and they have been tested in clinical trials.

DS-8895a is humanized anti-EphA2 monoclonal antibody recognizing the extracellular juxtamembrane region of the receptor. This antibody, produced by Daiichi-Sankyo, was able to increase the *in vitro* antibody dependent cellular cytotoxicity (ADCC) and to inhibit tumor growth in two xenograft mouse models with EphA2-positive human breast cancer and gastric cancer cells (Hasegawa et al. 2016). DS-8895 was also tested in patients with advanced or metastatic EphA2 positive solid cancers in order to evaluate tolerability, safety and pharmacokinetic profile (NCT02004717, [Satoh et al. 2019], NCT02252211).

Ifabotuzomab is anti-EphA3 monoclonal antibody made by Kalobios, which was tested in patient with refractory hematologic malignancies. The safety and tolerability were assessed by intravenous infusion administration of Ifabotuzumab in 64 patients and the most common toxic effects were transient grade 1 and 2 infusion reactions in 79% of patients whereas only 23% patients developed grade 3 adverse effects [Swords et al. 2016].

The treatment of leukemia xenograft mice with Ifabotuzumab had led to inhibition of cancer cells marrow infiltration, delay in tumor spread and increased in the latency of the disease, by disruption of the stromal tumor architecture [Vail et al. 2014; Charmsaz et al. 2016]. However, when Ifabotuzumab was tested in patients, clinical efficacy was displayed only for few patients.

Finally, PF-06647263 is an anti-ephrinA4 human antibody conjugated with calicheamicin which has showed a manageable safety a good pharmacokinetic profile in patient with advanced solid tumors. Although preliminary antitumor effects were reported in patients with pretreated triple negative breast cancer and ovarian cancer, the clinical study of PF-06647263 was ended due to the restricted results obtained after optimal exposure to anti-ephrinA4 human antibody [Garrido-Laguna et al. 2019].

An anti EphA2 and EphA3 antibody was developed and it showed promising *in vitro* results by reducing the clonogenicity and proliferation capacity of recurrent glioblastoma cells. The biological agent was also tested in orthotopic model of recurrent glioblastoma and the intracranial administration of the bispecific antibody determined the reduction of the tumor burden in mice. The mechanism of action of the EphA2/A3 antibody was related to decrease of both proteins from the surface of cancer

cells. The idea to develop of bispecific antibody arose from the observation that recurrent human glioblastoma cells enhanced the expression of EphA3, which maintains cancer cells in a stem-like state, and of EphA2 receptor. [Day et al. 2013] This latter is known to drive the tumorigenicity and to promote invasiveness of glioblastoma stem cells [Binda et al. 2012; Miao et al. 2015] Thus, targeting these two pro-oncogenic receptors would favour a more differentiated and less aggressive phenotype of glioblastoma cells [Qazi et al. 2018].

Soluble forms of Eph or ephrin proteins can activate, as dimer, or inhibit as monomer the forward, reverse signalling or both.

Several research groups have investigated the biological effect of soluble form of Eph receptors in tumor mouse model. Inhibition of tumor angiogenesis and tumor growth *in vivo* were reported for soluble EphA2-Fc and EphA3-Fc receptors [Dobrzanski et al. 2004; Cheng et al. 2003; Brantley et al. 2002] but outstanding results were observed with soluble monomeric EphB4 fused with human serum albumin, s-EphB4-HSA [Shi et al. 2012]. This new molecule led to significant tumor suppression and reduced tumor vessel density in transgenic murine tumor model [Djokovic et al. 2010]. S-EphB4-HSA showed its efficacy also in Malignant pleural xenograft models, where the 66% of mice receiving sEphB4-HSA displayed tumor volume reduction after 25 days of treatment. The outcome of EphB4-ephrin-B2 signalling blockage was the induction of mesothelioma cell apoptosis, reduction of tumor angiogenesis and down-regulation of PI3K and Src pathways. In addition, the combination of sEphB4-HSA with Bevacizumab revealed superior efficacy than single agent alone, with a complete tumor regression [Liu et al. 2013].

Moreover, a pilot phase Ib (NCT02495896) and phase IIa trials are ongoing to evaluate side effects and best dose of association s-EphB4-HSA with standard chemotherapy, and to investigate how s-EphB4-HSA with an anti-PD1 antibody work on treating patients with advanced or metastatic solid tumors [El-Khoueiry et al. 2016; Hugen et al. 2016].

Also soluble ephrins showed therapeutic efficacy in mice, in fact, injection of soluble ephrin-B2 into head and neck squamous cell carcinoma xenografted mice suppressed tumor growth by preventing leakage and the formation of further vessels [Kimura et al. 2009)].

Small molecules: Another approach to target Eph receptor is the use of agonist or antagonist small molecules which interact with the ephrin-binding pocket of Eph receptors. Small molecule antagonists hamper the activation of the receptor and thus the downstream cascade signalling by competing with ephrin ligands for the binding with Eph receptors. In the same way, they prevent the reverse signalling induced by ephrin ligand. Molecules such as these, may find application in conditions where receptor activation contributes to the pathology.

In this regard, different groups have pointed to identification of small molecules targeting the extracellular ligand-binding domain of Eph receptors, despite the large size and flexibility of the Eph-ephrin interacting surface. Few classes of binders for EphA and EphB have been discovered, including lithocholic and cholenic acid derivates and doxazosin. The latter compound is α1-adrenoreceptor antagonist which is also an EphA2 agonist. This molecule was able to reduced distal metastasis and prolonged survival of PC3 cells orthotopically injected in nude mice [Petty et al. 2012].

Lithocholic acid and its derivate UniPR129

Lithocholic acid (LCA) was found as a reversible, competitive antagonist of EphA2 receptor through High throughput screening campaign performed by University of Parma in 2011 (Fig.5). This secondary bile acid was a pan Eph inhibitor, being a promiscuous ligand of both family of Eph receptors, and it blocked the binding between EphA2 receptor and ephrin-A1 ligand with Ki value of 49μM. In cellular assays performed on PC3 cells, T47D cells and HT29 the compound dose dependently inhibited the ephrin-induced phosphorylation of Eph receptors without exerting cytotoxic effects and without affecting the kinase domain. Moreover, LCA was completely inactive against other kinases (EGFR, VEGFR, IRK or IGFR1), confirming its specificity for Eph receptors. Finally, LCA was able to inhibit PC3 cells retraction and rounding, two events that usually follow to ephrin-A1 induced EphA2 stimulation [Giorgio et al. 2011]. The discovery that LCA is a PPI-I has started a research program aimed to identify LCA derivates with improved binding affinity and inhibitory activity for EphA2. Structure-activity relationship demonstrated as the steroidal core of LCA placed itself into lipophilic channel of EphA2 and it established salt bridge with the Argine103, thus, mimicking the binding pose of ephrin-A1 to EphA2 [Tognolini and Lodola 2015]. The LCA structure was used as template and other compounds were designed and synthetized by chemists of University of Parma.

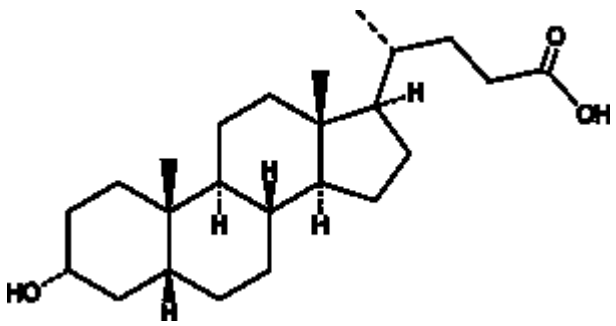


Figure 5: Lithocholic acid structure [Giorgio et al. 2011]

A remarkable potency improvement was reached with UniPR129, the L-homo tryptophan conjugate of LCA which turned out a potent competitive and reversible Eph antagonist [Hassan-Mohamed et al. 2014] (Fig.6). This compound showed to disrupt the EphA2-ephrin-A1 interaction with a Ki value of 390nM in ELISA binding assay, and to inhibit the ephrin-A1 induced EphA2 activation with IC_{50} value of 5μM in cell functional assay, without exerting cytotoxic effects. Moreover, UniPR129 exhibited *in vitro* anti-angiogenic property, mainly due to its ability to affect the migration of endothelial cells through interference with Eph-ephrin system. Nevertheless, successive pharmacokinetic studies revealed a poor bioavailability of the compound in mice. In fact, UniPR129 was endowed by high lipophilicity, low solubility and rapid degradation in mouse liver microsomes, properties which hampered the *in vivo* study.

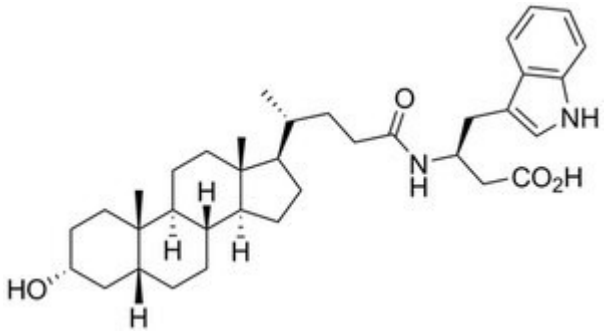


Figure 6: Structure of UniPR129 [Hassan-Mohamed et al. 2014]

AIM

It is well-known that the Eph-ephrin system is involved in several pathological processes, especially in cancer. Altered expression and function of Eph receptors and ligands have been observed in many tumors such as breast, colon, liver, prostate, and glioblastoma, and they have been related with malignant progression, tumor angiogenesis, metastasis, propagation and maintenance of tumor stem cell. Based on these findings, the idea to target this system as a new or alternative therapeutic strategy in oncology.

Different approaches to interfere with Eph/ephrin signalling have been explored over the years and some of them have reached clinical trials, showing promising results.

Since 2009, our research group has focused its attention on the development of small molecules able to hamper the Eph-ephrin binding by targeting the extracellular ligand binding domain of Eph receptor.

The identification of Lithocholic acid as a reversible, competitive antagonist of the Eph-ephrin system started a research campaign aimed to identify new molecules with higher affinity and potency. Thus, the Lithocholic acid structure was used as scaffold and through structure-activity relationship analysis new derivates have been synthesized and investigated for their ability to disrupt EphA2–ephrin-A1 binding.

Two main approaches have been adopted: 1) conjugation of lithocholic acid with linear alkyl amino acids [Incerti et al. 2017] 2) replacement of the hydroxylic group in position 3 of lithocholic acid with different substituents.

The first approach brought to the identification of the potent antagonist UniPR129, the L-homotryptophan conjugated of lithocholic acid, in 2014[Hassan-Mohamed et al. 2014]. However, UniPR129 suffered from poor solubility and low bioavailability in mice, features which have limited the *in vivo* study. For these reasons, two series of UniPR129 derivates were developed and synthesized through the combination of the structure-activity relationship (SAR) analysis and metadynamics (META-D) by Dott. Matteo Incerti and Prof. Alessio Lodola of Food and Drug Department at University of Parma [Incerti et al. 2017]. The first series resulted from the substitution of indole group in β-position of UniPR129 with other aromatic substituents with specific orientation and dimension (Fig. 7B). The second series of compounds came from the introduction of more hydrophilic substituents, as the carbamoyloxy group, instead of the hydroxylic group in 3α position of UniPR129. This modification allowed to retain the inhibitory potency and to improve the oral bioavailability, thus new compounds with 3α-carbamoyloxy group bearing different substituents on Nitrogen atom were developed and studied (Fig.7C).

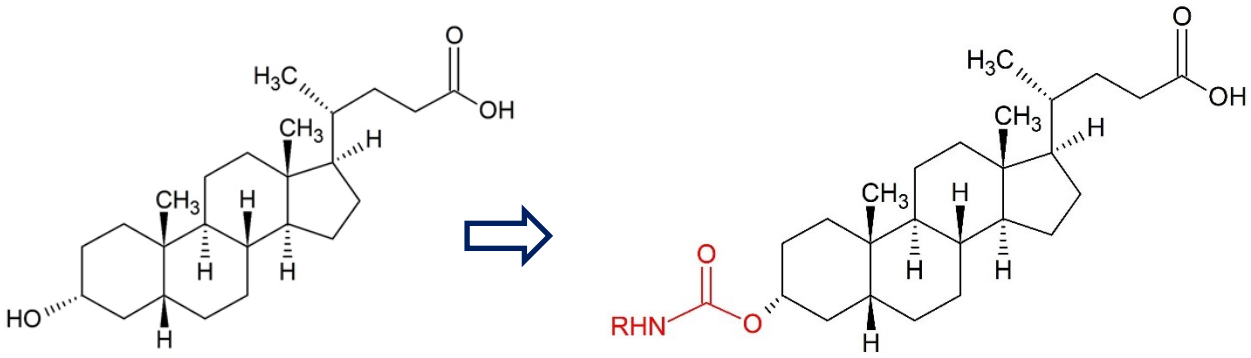
The last strategy was also applied on Lithocholic acid, leading to the synthesis of 3α N-substituted carbamates derivates (Fig.7A).

UniPR129 derivates together with 3α-carbamoyloxy Lithocholic acid derivates were pharmacologically characterized with the aim to identify potent Eph binders, interfering with the Eph/ephrin system and endowed by anti-tumoral properties.

As first step, all new synthetized molecules have been tested in displacement studies in order to select only the most potent. Then, these selected compounds were investigated for their ability to inhibit the EphA2 activation, cytotoxic effects and *in vitro* anti-angiogenic properties through functional assays performed on cells. Finally, only compounds, which have shown a good pharmacokinetic profile in mice, were tested in tumor animal models.

Figure 7: Graphical representation of compounds obtained from UniPR129 and from Lithocholic acid.

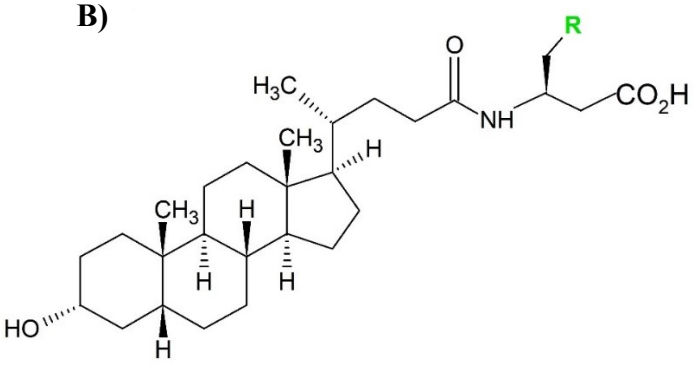
A)



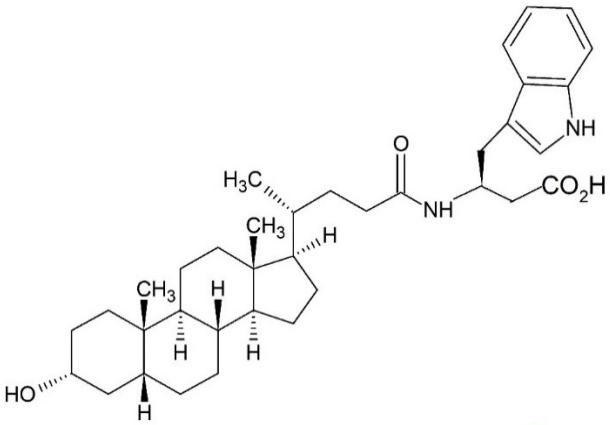
Lithocholic acid

3α- N substituted carbamoyloxy derivatives of Lithocholic acid

B)

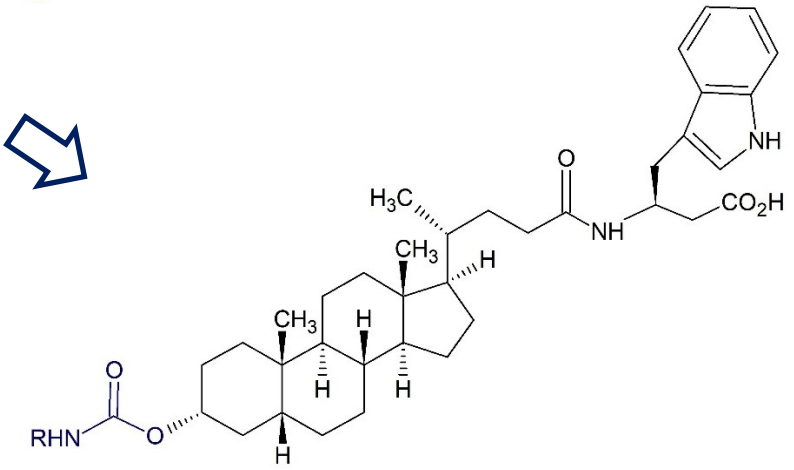


UniPR129 derivates with different aromatic substituents in β-position of amino acid



UniPR129

C)



3α-N substituted carbamoyloxy derivatives of UniPR129

MATERIAL AND METHODS

Cell cultures

PC3 human prostate adenocarcinoma cells (ECACC, Port Down, UK) were cultivated in Ham-F12 (Carlo Erba, Italy) with 7% fetal bovine serum (FBS, Euroclone, Milan, Italy) and 1% penicillin-streptomycin (Euroclone, Milan, Italy). HUVEC Human umbilical vein endothelial cells (Life Technologies, Waltham, MA, USA) were maintained in MEM 200 (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with LSGS kit (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 1% penicillin–streptomycin solution and 10% FBS. Cells were grown in a humidified atmosphere of 95% air, 5% CO_2 at 37°C.

Cell lysates

PC3 and HUVEC cells were seeded and growth in 6 or 12-well plates until they reached 70% confluence and then serum starved overnight. The day after, cells were treated with compounds, vehicle or standard drug and stimulated with the physiological agonist. After that, cells were rinsed with cold PBS and solubilized in lysis buffer, containing 1% NP-40 Alternative, 20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 10µg/mL Aprotinin, 10µg/mL Leupeptin. Lysates were collected, kept in ice for 15 minutes and then centrifuged at 2000g for 5 min.

The protein concentration of supernatant was determined by BCA protein assay kit (Thermo scientific, Waltham, MA, USA), standardized to 150µg/ml.

For EphA2 and EGFR phosphorylation evaluation, PC3 cells were treated in the following way:

1-pre-treatment with compounds, vehicle (DMS0 0.3%) or standard drug for 20 minutes

2-stimulation with ephrin-A1-Fc 0.25µg/ml for 20 minutes (for phosphoEphA2) or with EGF-Fc 10ng/ml for 10 minutes (for phosphor-EGFR). PBS was used as vehicle in this case.

For VEGFR2 phosphorylation, HUVEC cells were undergone to pre-treatment with compounds, vehicle (DMS0 0.3%) or standard drug for 20 minutes and to stimulation with 10ng/ml $VEGF_{165}$ for 5 minutes or with PBS.

Evaluation of EphA2, EGFR and VEGFR2 phosphorylation through ELISA ASSAY

EphA2, EGFR and VEGFR2 phosphorylation have been measured in cell lysates using DuoSet®IC Sandwich ELISA (R&D Systems,) following manufacturer's protocol. A 96 well ELISA high binding plate (Costar) was coated with specific capture antibody diluted in sterile PBS (0.2 g/l KCl, 298.0 g/l NaCl, 0.2 g/lKH2PO4, 1.15 g/l Na2HPO4, pH 7.4) at concentration of 4µg/ml and incubated overnight at room temperature. The day after, wells were rinsed 5 times with washing solution (PBS + 0.05% tween20, pH 7.4) and blocked for 1 hours with 300µl/well of blocking solution (PBS + 1% BSA) before the sample addition. Then, blocking solution was removed, wells were newly washed, and lysates were added at room temperature. After 2 hours, lysates were taken out and anti-phosphotyrosine antibody conjugated to HRP was added and incubated at room temperature for other 2 hours. The amount of phosphorylation was revealed using a colorimetric reaction read at 450 nm.

LDH assay

Cytotoxicity of the compounds was measured using CytoTox 96 Non-Radioactive Cytotoxicity Assay, following the manufacturer's protocol (Promega). PC3 and HUVEC cells were seeded in 96-well plates at a density of 10^5cells/ml, the day after they were serum starved and treated with compounds or lysis buffer for 2 or 16 hours respectively. The released LDH in supernatants was evaluated by incubating the reactive solution for 30 minutes. In presence of LDH, 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. The cytotoxic effect was calculated with the following formula:

$$\left(\frac{\text{absorbance of cells treated with compounds - absorbance of cells treated with vehicle}}{\text{absorbance of cells treated with lysis buffer - absorbance of cells treated with vehicle}}\right) *100$$

ELISA EphA2 binding assay

96 well ELISA high binding plate (Costar) was incubated overnight at 4°C with 100µl/well of 1µg/ml EphA2-Fc (R&D) diluted in sterile PBS, washed 3 times with wash buffer and blocked with 300µl/well of blocking buffer (PBS + 0.5% BSA) for 1 hour at 37°C. After that, wells were rinsed again 3 times with wash buffer and compounds were added to the wells at tested concentrations and incubated at 37°C for 1 hour. Biotinylated ephrin-A1-Fc (R&D Systems) was added for 4 hours at

20ng/ml in displacement studies whereas in a range of 1-2000ng/ml for 5 hours in saturation studies. After this time, wells were washed 3 times and incubated for other 20 minutes at room temperature with 100µl/well streptavidin-HRP (Sigma) solution (0.05µg/ml in PBS with 0.5% BSA, pH 7.4), washed again for three times and finally 100µl/well of TMB solution (tetra-methylbenzidine (Sigma) reconstituted in stable peroxide buffer (11.3 g/l citric acid, 9.7 g/l sodium phosphate, pH 5) supplemented with 0.02% H_2O_2 30% m/m in water) was added. 100µl/well of 3N HCl were used to stop the reaction and the absorbance was measured at 450 nm using an ELISA plate reader (Sunrise, TECAN, Switzerland).

Reversibility binding assay

To evaluate the reversibility of compounds binding to EphA2 receptor, displacement studies were performed as described. 1µg/ml EphA2-Fc diluted in sterile PBS was incubated overnight at 4°C onto 96 well ELISA high binding plate (100µl/well). The next day, wells were washed, blocked with block buffer and rinsed again as before indicated for EphA2 displacement experiment. After that, wells were incubated with tested compounds at biotinylated ephrin-A1-Fc displacing concentrations at 37°C. 1 hour later, compounds were washed away from some wells before adding 20ng/ml of biotinylated ephrin-A1-Fc for 4 hours. After washout and incubation with Streptavidin-HRP solution (100µl/well) for 20 minutes at room temperature, wells were washed and incubated for other 20 minutes with TMB solution (100µl/well). The reaction was arrested with 3N HCl (100µl/well) and the absorbance was read at 450nm using an ELISA plate reader.

Selectivity binding assay

To assess the selectivity of compounds, 100µl/well of 1µg/ml EphA (A1-A8) and EphB (B1-B4, B6) receptors diluted in sterile PBS were incubated overnight at 4°C onto a 96 well ELISA high binding plate. The day after, wells were washed with wash buffer and blocked with blocking solution (300µl/well) at 37°C. After 1 hours, wells were rinsed again, and compounds were incubated at 37°C for 1 hour. Then, biotinylated ephrin-A1-Fc (R&D) and biotinylated ephrin-B1-Fc at their K_D concentration were added for 4 hours and used toward EphAs or EphBs respectively. After this time, wells were washed and incubated with 100µl/well streptavidin-HRP solution for 20 minutes at room temperature, washed again and finally incubated with 100µl/well of TMB-solution. The reaction was stopped by adding 100µl/well of 3N HCl and the absorbance was measured at 450 nm using an ELISA plate reader.

Inhibition constant (K_i) and Inhibitory concentration IC_{50} determination

The IC_{50} value was calculated using nonlinear regression. The dissociation constant K_D and the apparent dissociation constant K_D' values of the saturation curves in absence or in presence of compounds were calculated using non-linear regression analysis and employed to determine the Log [DR-1]. Then, Schild plot was drawn [Arunlakshana and Schild 1959], where Log_{10}[DR-1] is a function of the negative Log_{10} of the inhibitor concentration and used to calculate the Ki and the Hill's coefficient of compounds.

Tube formation assay

In 96 well-plate for tissue culture, a thin layer of gel on the bottom of wells was made by coating with 40µl/well BD Matrigel (BD Biosciences Bedford, MA, USA) for 30 minutes at 37°C. Then HUVEC cells were seeded on Matrigel at concentration of $2*10^4$cells/well. Cells were treated with compounds or with vehicle (0.3% DMSO) and after 16 hours, HUVEC were fixed with 3.7% formaldehyde (ROMIL, Waterbeach, Cambridge, UK,) for 15 min at room temperature. Photographs of each well were taken through a digital camera mounted on a microscope (Eurotek, Orma, Italy) and the number of polygons formed was counted. The results were expressed as the ratio between the number of polygons formed by the cells treated with the compounds and the untreated cells.

Pharmacokinetic studies

Animals were housed, handled, cared and euthanised accordingly to the European Community Council Directive 2010/63/UE and Italian regulation (DL 26/2014).

The bioavailability of compounds was assessed through oral administration of compounds suspended in 0.5% methylcellulose MC (Sigma-Aldrich, St. Louis, MO, USA) to 8 weeks old C57BL/6 male mice, (Charles River Laboratories, Milan, Italy). Compounds were dosed at 30 mg/kg and each group was formed at least by 2 mice. Blood samples were collected via tail puncture at the different time-points, from 0 to 1440 minutes. Then samples were centrifuged (2000g, 4°C, 15 min) and plasma stored at -20°C until use. Compounds were measured by HPLC-ESI-MS/MS employing a Thermo Accela UHPLC gradient system coupled with a Thermo TSQ Quantum Access Max triple quadrupole mass spectrometer equipped with a heated electrospray ionization (H-ESI) ion source. Xcalibur 2.1

software (Thermo Italia, Milan, Italy) was used for sample injection, peaks integration, and plasma level quantification.

In vivo experiments

Animal experiments received the approval of local Animal Care Committee and Italian Ministry of Health and mice were used in compliance with European Community Council Directive 2010/63/UE and Italian regulation (DL 26/2014).

PC3 xenograft mice

Animal experiments received the approval of local Animal Care Committee and Italian Ministry of Health and mice were used in compliance with European Community Council Directive 2010/63/UE and Italian regulation (DL 26/2014).

6 weeks old BABL /c nude mice (Charles River, Milan, Italy) received two subcutaneous flank injections of 1×10^6 PC3 in 200µl Matrigel mixture. After the injection, tumor growth was monitored 2 times for week by measuring tumor diameters with a calliper. Tumor volume was calculated with the following formula: volume = $(d^2 \text{ x } D)/2$, where d and D are the shortest and longest diameters, respectively.

When mice developed tumor with a volume of 200 mm^3, they were randomised in 4 groups: UniPR139, UniPR502, control, and Sorafenib. 30mg/Kg of UniPR139 or UniPR502 and 2.5 mg/kg Sorafenib suspended in Methocel 0.5% were daily orally administered. The control group received only the vehicle. The experiments were carried out for 25 days, until the control group developed tumors with critical volumes for animal welfare laws. At the end of the study, all mice were sacrificed by carbon dioxide inhalation and tumors were subsequently excised, measured and weighted.

Apc^{Min}/J mice

5 weeks old C57BL/6J-ApcMin/J male mice were purchased from Jackson Laboratory (Bar Harbor, ME USA), a strain highly susceptible to spontaneous intestinal adenoma formation [Dopeso et al. 2009]. They were weighted and randomly subdivided in two groups:

-control group, with mice receiving the vehicle (Methocel 0.5%) through gavage

-UniPR129 group, where 30mg/Kg of UniPR129 suspended in Methocel 0.5% was oral administered to mice every other day. The experiment was carried out for 8 weeks and all mice were sacrificed by

carbon dioxide inhalation at the end of the study. The weight of the mice and the presence of blood in the stool were monitored once a week for the whole duration of experiment.
Blood samples were collected via intracardiac injection and used for evaluation of haematological parameters. The whole intestine was resected, washed with sterile PBS and fixed with 4% formalin. After 24 hours, the intestine was longitudinally divided in 6 portions (1-duodenum, 2-jejunum, 3-ileum, 4-caecum, 5-colon, 6-rectum) and each portion was embedded in paraffin and sectioned at 5µm with microtome (Leica). Slides were dried at 37°C for 24hours, then deparaffinized in xylene and rehydrated with decreasing concentration of alcohol.
Hystological slides were examined with Nikon Eclipse E800 microscope (Nikon Corporation, Japan) and photographs of sections were taken at 2x 4x, 10x, and 20x with Camera Nikon DIGITAL SIGHT attached to the microscope. Pictures were analysed through ImageJ in order to display and classify dysplastic, iperplastic and neoplastic lesions on mucosa of intestine.

Statistical analysis

Data are generally the means of at least three independent experiments ± standard deviation.
One-way ANOVA followed by Dunnet's post test was performed in phosphorylation assays, LDH assay, tube formation assay and to analyse the tumor weight in PC3 xenograft mice. Haematological and blood chemistry parameters were undergone to One-way ANOVA followed by Dunnet's post test or t-test to compare the control group to treatment groups in of PC3 xenograft mice and Apc^{Min}/J mice, respectively. Mann-Whitney test was carried out to compare tumor diameter and tumor area in Apc^{Min}/J mice. Numbers of adenomas in intestine sections and differences in dimensional distribution of tumor diameters in ileum were evaluated by Two-way ANOVA followed by Bonferroni's post-test. *$p<0.05$ **$p<0.01$ ***$p<0.001$

RESULTS

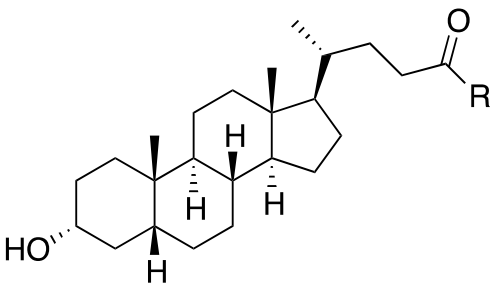
Preliminary Data

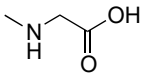
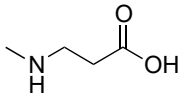
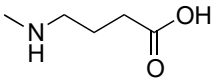
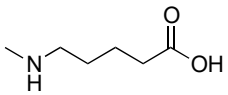
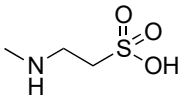
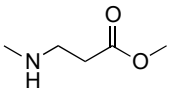
The discovery that LCA is able to prevent the interaction between EphA2 receptor and ephrin-A1 ligand, has initiated the research of derivates with higher potency and affinity for EphA2 receptor.
To do that, the LCA structure was used as template and it was thought:
-to conjugate the carboxylic acid with linear alkyl amino acids [Incerti et al. 2017];
-to replace the hydroxylic group in position 3 with different substituents.
The goal of this study was to explore LCA structure in order to find the best substituent to improve the binding to EphA2 receptor.

Conjugation of LCA with linear alkyl amino acids

The conjugation of the carboxylic group with the glycine increased almost half the potency for inhibition of EphA2–ephrin-A1 binding, even if a stronger improvement was obtained by elongating the alkyl chain between the amide and terminal carboxylic group. The IC_{50} value of β-alanine conjugated LCA (compound 3) was 29μM compared to 79μM of LCA. Conversely, a further lengthening of the chain led to a loss in the inhibitory potency. Also, the replacement of the carboxylic acid with a sulfonic acid (compound 6) did not bring to a gain in the inhibitory potency (Table 1) confirming the importance of a free carboxyl group for the interaction with EphA2.
Given these results, it was thought to generate a series of derivates of β-alanine conjugates of LCA bearing different substituents in β–position of the amino acid and to evaluate their impact on the ability to bind the EphA2 receptor (Table 2).
The introduction of small aliphatic branched chain (compounds 9) or aromatic groups as phenyl and benzyl (compounds 11-12) slightly improved the potency, instead a remarkable result emerged by introducing a bigger aromatic substituent with two aromatic rings. The presence of benzyl-imidazole (compound 14) group allowed to obtain a very potent compound featured by an IC_{50} in the low micromolar. That would seem to suggest that aromatic substituents with a specific orientation and dimension are important to get compounds capable to establish strong bonds with EphA2.

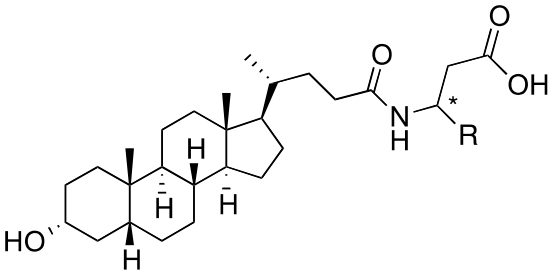
Table 1: IC_{50} values of LCA derivates

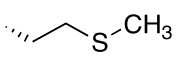
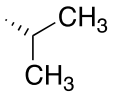
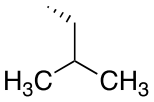
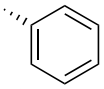
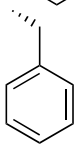
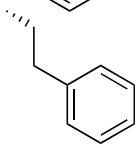
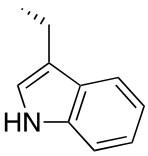
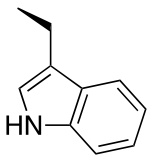


Compound	-R	IC_{50} (µM) ±95% C.I.
1 (LCA)		79 (67-93)
2		49 (42-58)
3		29 (23-36)
4		inactive
5		inactive
6		72 (53-100)
7		inactive

Data from Incerti et al., 2017

Table 2: IC_{50} values of β-alanine conjugates of LCA bearing different substituents in β position.



Compound	-R	IC_{50} (μM) ±95% C.I.
8		83 (50-138)
9		10 (6.6-16)
10		inactive
11		14 (10-20)
12		18 (12-26)
13		Inactive
14 **UniPR129**		0.9 (0.8-1.1)
15		26 (18-37)

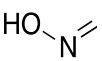
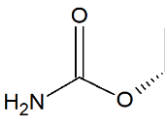
Data from Incerti et al., 2017

Replacement of 3α-hydroxyl group of LCA and UniPR129 with more polar substituents

As before mentioned, another strategy was to replace the hydroxylic group in 3α position of LCA, by inserting more hydrophilic group and to see how it could impact the inhibitory potency. This idea derives from previous observation [Incerti et al.2017] that the replacement of the 3α-hydroxylic group of UniPR129 with a more polar one as the 3α-carbamoyloxy group slightly decreased the IC_{50} and improved the pharmacokinetics properties of the compound. The removal (compound 17), the inversion of the chiral center (compound 16) and the oxidation (compound 18) of the 3α-hydroxy group were unsatisfactory in any case (Table 3).

Based on these results, two series of N-primary carbamates, one deriving from LCA and the other from UniPR129, were synthetized and evaluated for their ability to prevent ephrin-A1 binding to EphA2 trough an ELISA binding assay (Table 4).

Table 3: IC_{50} of UniPR129 derivatives substituted in position 3, obtained from EphA2-ephrin-A1 displacement experiments.

Compound	-R	IC_{50} (μM) ±95% C.I.
UniPR129 **14**		0.91(0.80-1.1)
16		17 (13-24)
17		28 (22-35)
18		28 (22-36)
19		3.1 (2.8-3.6)
UniPR502 **20**		0.80 (0.51-0.98)

Data from Incerti et al., 2017

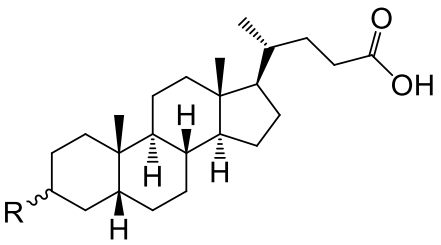
Testing these two series of compounds, it emerged that LCA derivatives were particularly sensitive to the introduction of carbamoyloxy group in position 3. That was mainly evident when a butyl (APO164), hexyl (APO184), cyclohexyl (APO 170) chain were inserted on the nitrogen of carbamoyloxy group, allowing to obtain compounds with an IC_{50} value around 3μM, 25fold less of their parental compound.

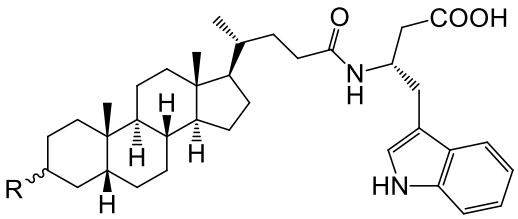
If for LCA derivates these structure modifications resulted in an overall dramatic increase of the potency, it was not the same for UniPR129 derivates. The introduction of long linear chains (PCM511), of iso-propyl (PCM506) and phenyl group (PCM504) on the Nitrogen was detrimental for the binding of EphA2 receptors, whereas smaller substituents allow to retain the same range of potency of their parental compound.

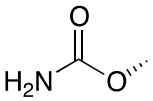
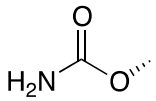
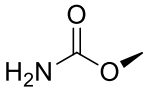
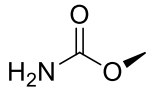
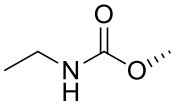
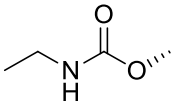
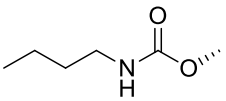
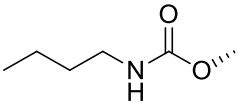
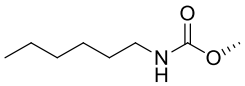
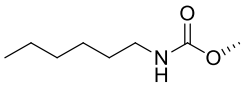
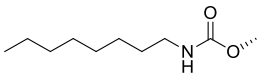
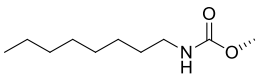
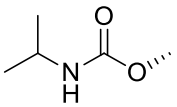
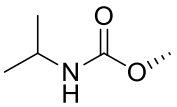
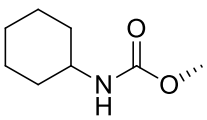
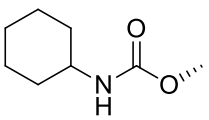
It was also interesting to see as the inversion of the chiral center of N-carbamoyloxy group was detrimental for UniPR129 derivates (PCM 149), instead, it was tolerated for LCA derivates (PCM147).

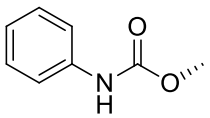
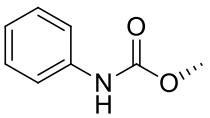
At the end of these studies, the most potent compounds of both series were selected and further binding studies were carried out, in order to examine the mechanism of binding for EphA2 receptor. As concerns LCA derivates, compounds with IC_{50} value below 3μM were chosen, whereas for UniPR129 derivates it was decided to test compounds having an IC_{50} below 1μM.

Table 4: IC_{50} values for 3-Carbamoyloxy LCA derivatives (left column) and for 3-Carbamoyloxy UniPR129 derivatives (right column) obtained from EphA2-ephrin-A1 displacement experiments.





Compound	-R, IC_{50} (μM) ±95% C.I.	Compound	-R, IC_{50} (μM) ±95% C.I.
3 PCM413	 17 (8.7-35)	**12** UniPR502	 0.8 (0.68-0.97)
4 PCM147	 9.1 (6.1-14)	**13** PCM149	 5.2 (3.0-9.1)
5 APO155	 30 (20-44)	**14** PCM505	 0.95 (0.60-1.5)
6 APO164	 3.7 (2.9-4.6)	**15** PCM507	 1.1 (0.7-1.8)
7 APO184	 3.3 (2.4-4.5)	**16** PCM510	 1.5 (1.2-2.0)
8 APO198	 4.5 (3.2-6.3)	**17** PCM511	 15 (13-17)
9 APO159	 12 (8.9-14)	**18** PCM506	 7.1 (3.9-13)
10 APO170	 2.7 (2.1-3.4)	**19** PCM508	 1.2 (0.94-1.5)

11 PCM142	 38 (21-68)	**20** PCM504	 5.2 (2.9-9.4)

Molecular mechanism of binding of UniPR502 and PCM505 for EphA2 receptor

The mechanism of binding of UniPR502 and PCM505 was analysed through saturation curves of biotinylated ephrin-A1 in presence of escalating concentrations of two compounds. For each curve the dissociation constant (K_D) or the apparent K_D were calculated and employed to draw the Schild plot, where log [DR-1] is the function of –log10 [inhibitor] [Arunlakshana e Schild 1959].

UniPR502 shifted in a concentration-dependent manner the saturation curves of biotinylated ephrin-A1 without affecting the B_{max}, suggesting a competitive binding (Fig. 8A). This idea was confirmed by the Hill coefficient value, deriving from the Schild analysis, which was equal to 0.99 and thus, by giving the proof of a competitive mechanism of binding for EphA2 (Fig. 8B). PCM505, instead, displayed a surmountable antagonism but the right curve shift was not proportional to antagonist concentrations (Fig. 8C) as also confirmed by Schild plot whose slope was significantly different from unity (data not shown). These results did not allow to explain the molecular mechanism of binding of PCM505 and further studies are necessary to clarify that.

Both compounds turned out being reversible binders for EphA2 receptor, as demonstrated by the restored biotinylated ephrin-A1 binding in wells after inhibitor wash out (Fig. 8D).

The next step was to evaluate the selectivity of UniPR502 and PCM505 towards all the members of two families of receptors, EphA and EphB. To do that, biotinylated ephrin-A1 or ephrin-B1 were used at their K_D.

UniPR502 did not discriminate between the two family of receptors and bound promiscuously to both subclasses, thus, acting as pan-inhibitor (Fig. 9A).

Conversely, PCM505 showed to mainly bind the EphA receptors family and in particular the EphA2 subtype. On the same time, it was inactive towards some receptors of both family as EphA1, EphA7, EphB1, EphB4 and EphB6 (Fig. 9B).

Figure 8: A) Saturation curves of biotinylated ephrin-A1-Fc on EphA2 built in presence of increasing concentration of UniPR502; B) Schild Plot of UniPR502; C) Saturation curves of biotinylated ephrin-A1-Fc on EphA2 built in presence of increasing concentration of PCM505; D) Reversibility assay: EphA2-ephrin-A1 binding in presence of 10μM of compounds with or without washing with PBS. Data are the means of at least 3 independent experiments. Standard Deviation (SD) was omitted for clarity

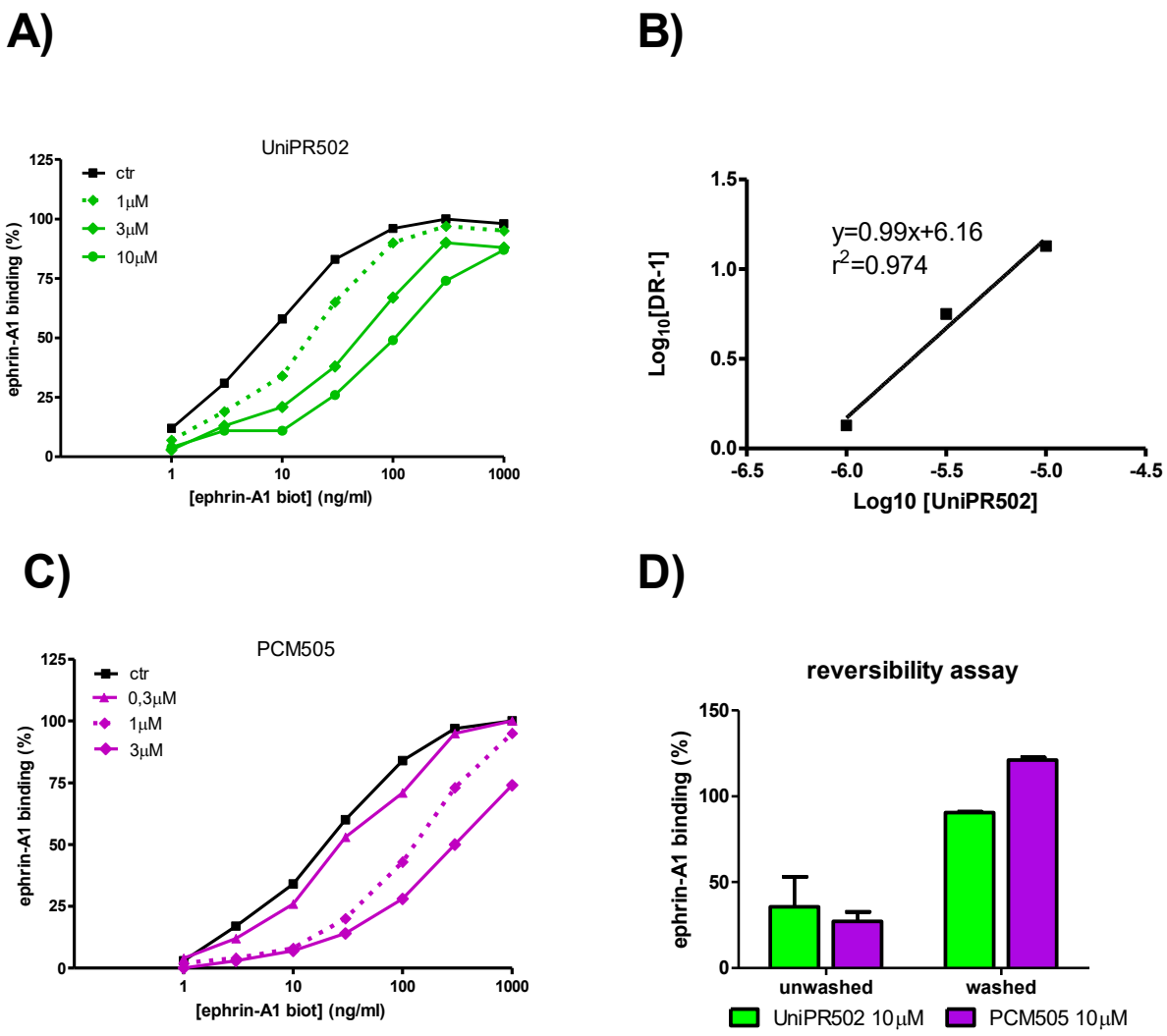
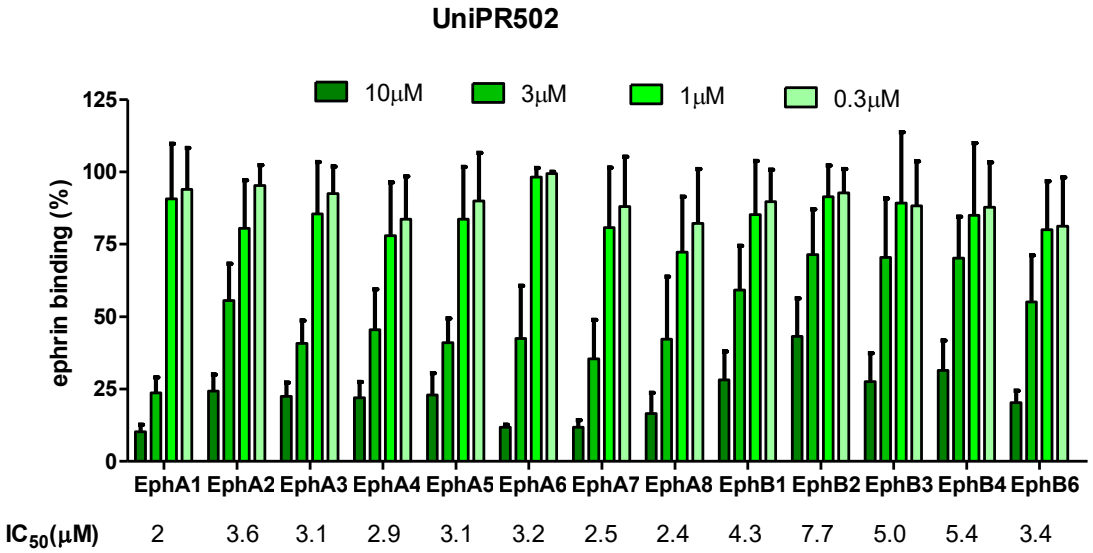
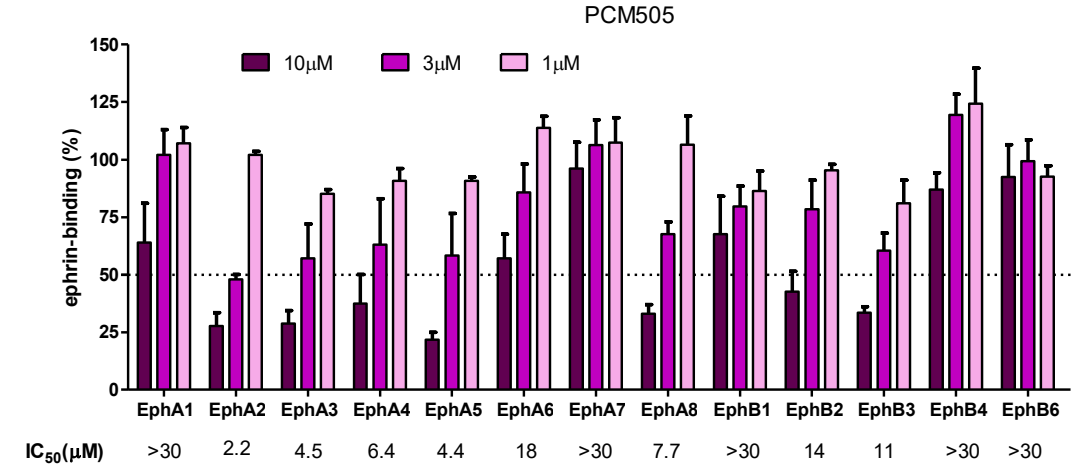


Figure 9: Selectivity assay: Displacement curves of biotinylated ephrin-A1/B1 from immobilized EphAs-Fc or EphBs-Fc receptors by UniPR502(A) or PCM505 (B). Data are the means of at least 3 independent experiments ± SD

A)



B)



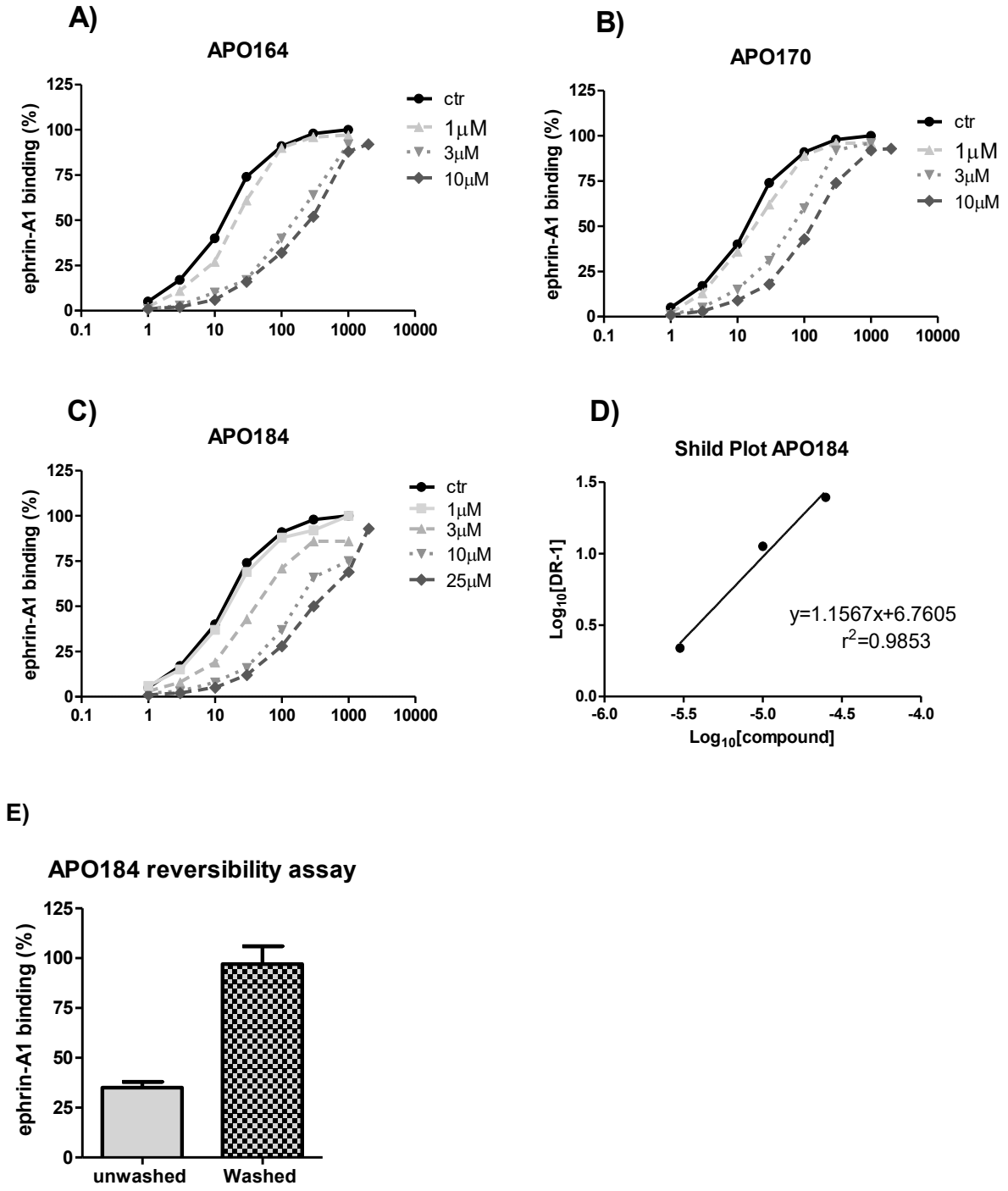
Molecular mechanism of binding of 3-Carbamoyloxy LCA derivatives for EphA2 receptor

Among the series of 3-Carbamoyloxy LCA derivatives tested in displacement assays, the most potent compounds were further characterized. The saturation curves for EphA2–ephrin-A1 binding in presence of increasing concentrations of APO164, APO184 and APO170 were plotted and the K_D or apparent K_D were calculated for each curve and used to draw the Schild plot.

Although the trend of saturation curves in presence of APO164 or APO170 seemed to suggest a competitive binding, the interpolated regression line showed a slope value of 1.35 ($r^2 = 0.84$) and 1.34 ($r^2 = 0.89$), respectively, indicating a stoichiometry ratio different from 1:1 and, thus, a diverse mechanism of binding.

Conversely, APO184 confirmed to competitively and reversibly bind the EphA2 receptor, in fact, the slope of the interpolated regression line was 1.15 ($r^2 = 0.98$), a value comprised between 0.8 and 1.2 (Fig. 10 C, D, E). Finally, a Ki value of 1.43μM was calculated for APO184.

Figure 10: A) Saturation curves of biotinylated ephrin-A1-Fc on EphA2 built in presence of increasing concentration of APO164; B) Saturation curves of biotinylated ephrin-A1-Fc on EphA2 built in presence of increasing concentration of APO170; C) Saturation curves of biotinylated ephrin-A1-Fc on EphA2 built in presence of increasing concentration of APO184; D) Schild Plot and coefficient Hill of APO184; E) Reversibility assay: EphA2-ephrin-A1 binding in presence of 10μM APO184 with or without washing with PBS. Data are the means of at least 3 independent experiments. SD was omitted for clarity



Identification of UniPR129 derivates bearing a different substituent in β-position of the amino-acid moiety

As mentioned above, UniPR129 is a potent and competitive inhibitor of the EphA2-ephrin-A1 binding, which has shown to suppress *in vitro* angiogenesis through the block of the ephrin-A1 induced EphA2 activation [Hassan-Mohamed et al. 2014].

Nevertheless, these promising results were hampered by the poor solubility and rapid degradation in mouse liver microsomes of the compound which have limited the *in vivo* study. For these reasons, through the combination of the structure-activity relationship (SAR) analysis and metadynamics (META-D) [Incerti et al. 2017] new compounds were synthesized and tested in displacement assays. These compounds differed each other for the aromatic substituent in β-position of the amino-acid moiety. Among them, UniPR139 emerged as alternative promising compound, which owned a benzothiophene instead of an indole (Fig. 11). This modification brought on the one hand to moderate reduction of the inhibitory potency but on the other hand an improvement of the physiochemical properties. Analyzing UniPR139 in displacement assay, it dose-dependently displaced biotinylated ephrin-A1 from the immobilized EphA2 with an IC_{50} 2-times higher than parental compound, but still in the low micromolar range (Fig.12A). Moreover, as well as UniPR129, UniPR139 bound reversibly the EphA2 receptor. When displacement studies were repeated by incubating 10μM of compound for 1 hour and washing some of the wells before the addition of biotinylated ligand, the displacement of biotinylated ephrin-A1 was detected only in the unwashed wells (Fig. 12B).

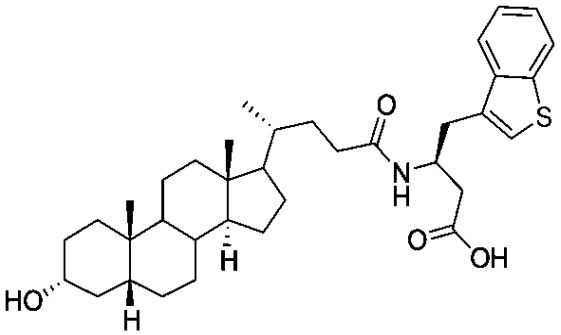
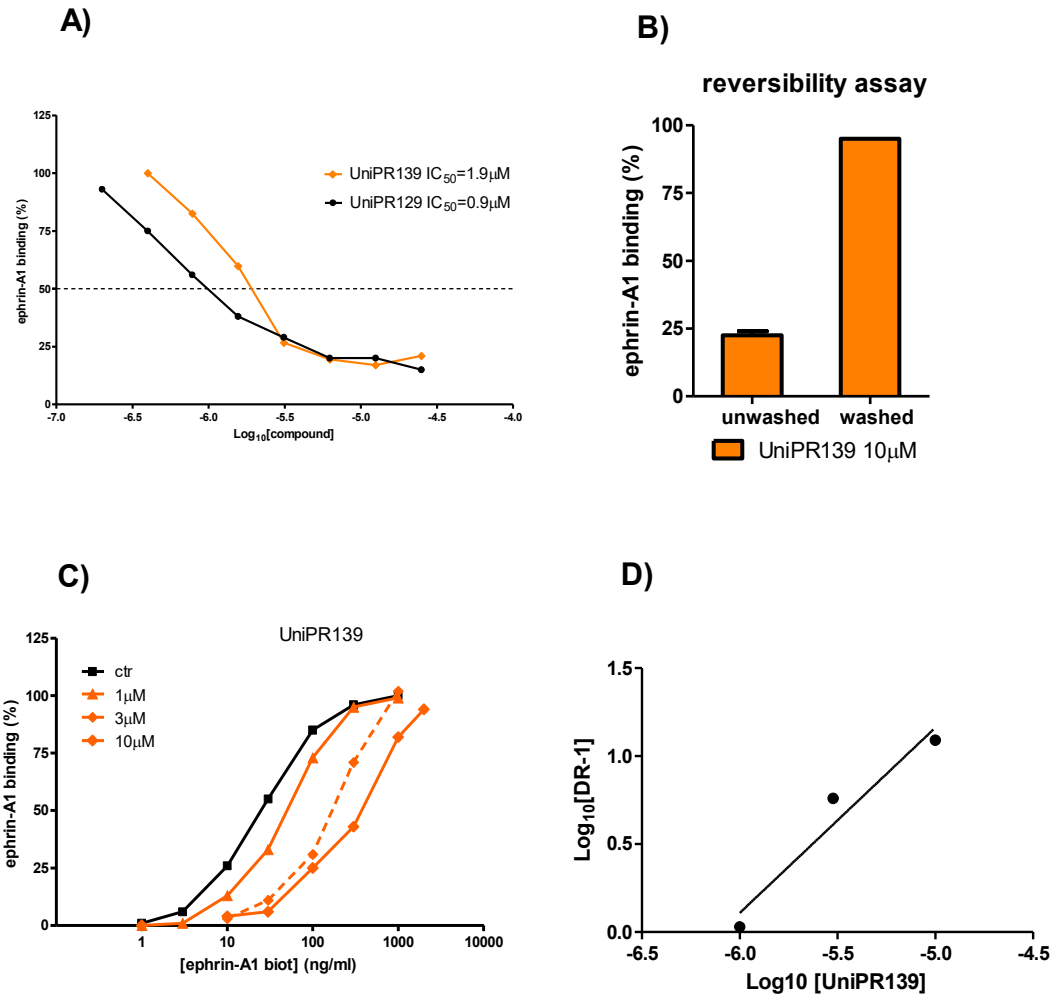


Figure 11: Structure of UniPR139.

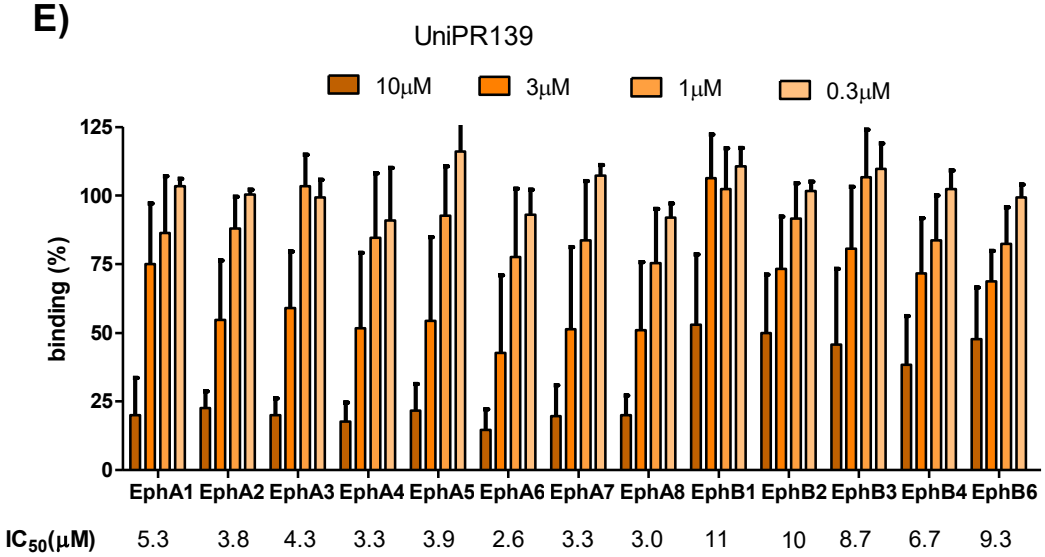
Saturation studies showed a competitive binding for UniPR139, which was able to shift saturation curves toward right in a concentration-dependent manner, preserving the B_{max} (Fig. 12C). That was also corroborated by the Hill coefficient, calculated trough Schild analysis, whose value was 1.06 (r^2=0.954). The pK_i resulting from the intersection of the regression line with the X axis showed values of -6.022 for UniPR139 (K_i=950nM) (Fig. 12D).

Finally, the selectivity of the binding through the two subfamilies of Eph receptors was investigated. The ability to inhibit ephrin binding to all EphA and EphB receptors was analyzed using biotinylated ephrin-A1-Fc or biotinylated ephrin-B1-Fc respectively at their K_D concentrations. UniPR139 was not able to discriminate between EphAs and EphBs receptors, although it showed a slight preference for A-subfamily (Fig.12E). The behaviour of the compounds was similar to what previously observed for the parental compound UniPR129.

Figure 12: A) Displacement curves of 20 ng/ml biotinylated ephrin-A1-Fc on immobilized EphA2-Fc ectodomain by increasing concentrations of UniPR129 and UniPR139; B) Reversibility assay: EphA2-ephrin-A1 binding in presence of 10μM UniPR139 with or without washing with PBS; C) Saturation curves of biotinylated ephrin-A1-Fc on EphA2, built in presence of increasing concentration of UniPR139; D) Schild Plot of UniPR139; E) Selectivity assay: Displacement curves of biotinylated ephrin-A1/B1 from immobilized EphAs-Fc or EphBs-Fc receptors by UniPR139. Data are the means of at least 3 independent experiments. SD was omitted for clarity in A and C.



E)



	EphA1	EphA2	EphA3	EphA4	EphA5	EphA6	EphA7	EphA8	EphB1	EphB2	EphB3	EphB4	EphB6
IC_{50}(μM)	5.3	3.8	4.3	3.3	3.9	2.6	3.3	3.0	11	10	8.7	6.7	9.3

UniPR139, UniPR502, PCM505 and APO184 act as EphA2 antagonists

Binding studies were useful to select the most promising compounds. Molecules featured by an IC50 near or below the micromolar range and acting as competitive inhibitors, were chosen for further studies. Thus, UniPR139, UniPR502, PCM505, APO184 were tested in *in vitro* functional studies. First of all, it was verified their ability to inhibit the EphA2 activation induced by ephrin-A1 in PC3 cells (human prostate adenocarcinoma cells). This cell line was used as experimental model due to the natural over-expression of EphA2 receptor [Miao et al. 2000].

According with binding results, UniPR139 and PCM505, inhibited in a concentration-dependent manner the EphA2 phosphorylation induced by ephrin-A1-Fc 0.25μg/ml, showing an inhibitory activity very close to the one observed in cell-free displacement assays. Conversely, UniPR502 and APO184 turned out less active, displaying an IC_{50} of 6μM and of 16μM respectively, which were almost 8-fold and 3-fold higher than binding studies (Fig. 13A). In the same experimental conditions, the multikinase inhibitor dasatinib, used as reference compound, completely blocked EphA2 phosphorylation at 1μM.

As expected, none of the compounds showed agonistic properties, in fact, no signal was detected when PC3 cells were treated only with compounds without ligand stimulation, confirming that they act as antagonist (data not shown).

Because an inhibition of the EphA2 activation could be ascribed to a non specific cytotoxic effect, LDH assay was performed on PC3 cells, by testing compound at concentrations capable to block the phosphorylation of the receptor. The amount of released LDH was assessed after 2h of incubation with compounds and none of them caused an appreciable release (Fig. 13B). These results corroborated that compounds are able to directly interfere with the EphA2 activation. At the end of phosphorylation assays, it was decided to keep the study solely of UniPR139, UniPR502, PCM505 and APO184 was discarded, due to its poor potency and efficacy to inhibit the activation of EphA2 (Table 5).

*Figure 13: A) Phosphorylation of EphA2 in PC3 cells. Cells were pretreated for 20 min with 0.3% DMSO or with the compounds at the indicated concentrations and then stimulated for 20 min with ephrin-A1-Fc 0.25µg/ml. Phospho-EphA2 levels are relative to ephrin-A1-Fc + DMSO. B) relative levels of LDH after 2h of incubation with compounds. One-way ANOVA followed by Dunnet's post-test was performed to compare ephrin-A1-Fc + DMSO to all the other columns (A) and control (0% column not shown) to all other columns (B) *p < .05, **p < 0.01, *** p<0.001. Data are the means of at least 3 independent experiments±SD*

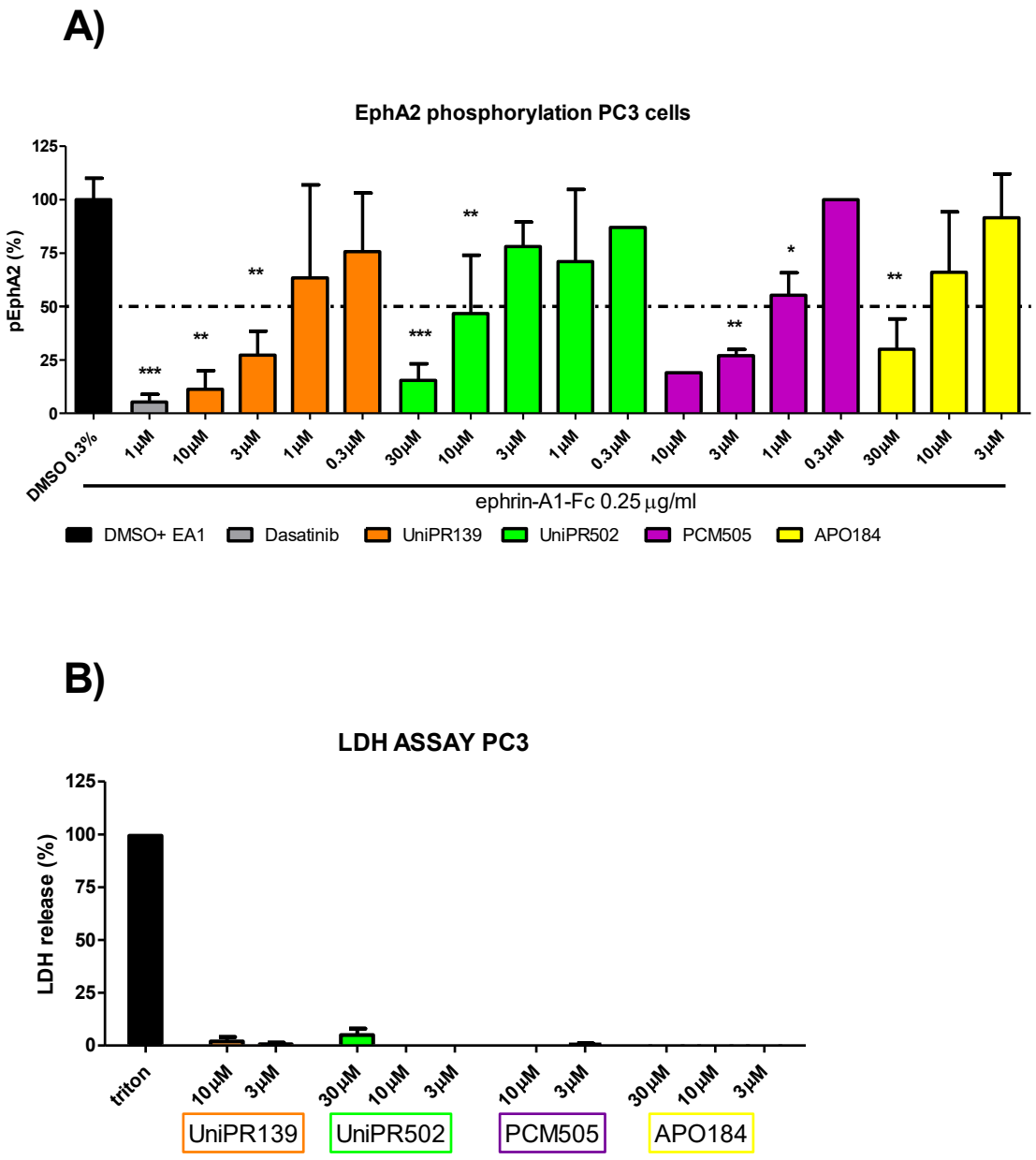


Table 5: Comparison among IC_{50} and Ki calculated in cell-free displacement assay and in PC3 cells.

	EphA2/ephrin-A1 binding		**EphA2 phosphorylation PC3 cells**
	IC_{50} (μM) (95% conf limits)	K_i (μM) (95% conf limits)	IC_{50} (μM) (95% conf limits)
UniPR129	0.95 (0.68-1.30)	0.39 (0.31-0.45)	5.0 (3.5-7.1)
UniPR139	1.8 (1.5-2.3)	0.95 (0.65-1.25)	2.0 (1.2-3.3)
UniPR502	0.80 (0.68-0.97)	0.75 (0.58-0.93)	6.41 (4.43-9.28)
PCM505	0.95 (0.60-1.5)	0.33(0.26-0.42)*	1.8 (0.6-4.9)
APO184	3.3 (2.4-4.5)	1.43 (1.14-1.71)	15.8 (6.2-40.4)

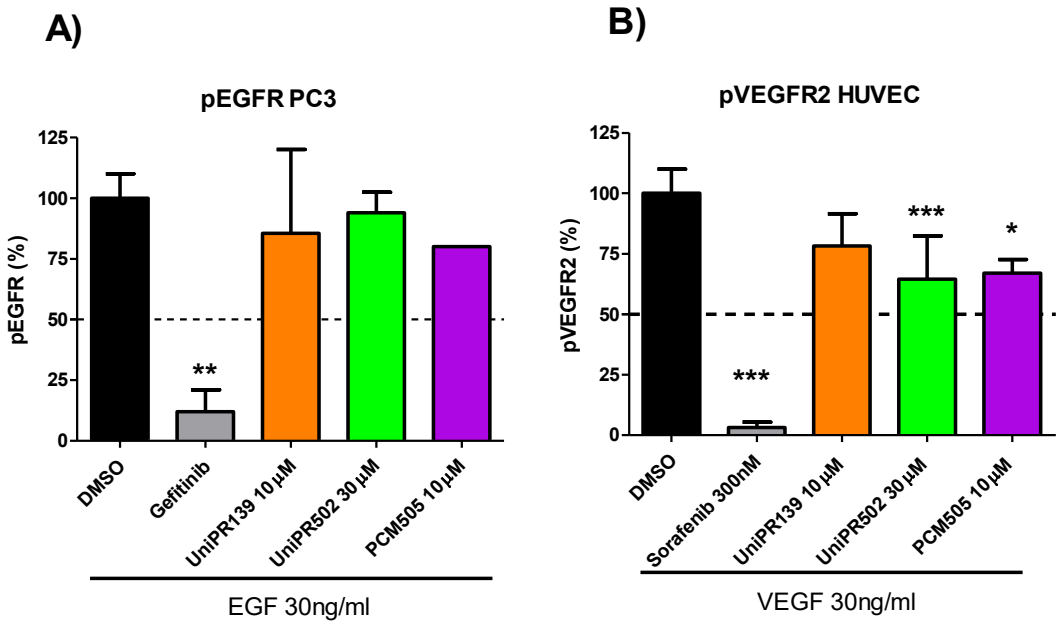
*Roughly estimated considering a competitive antagonism

Evaluation of OFF TARGET effects of UniPR139, UniPR502 and PCM505

After that, we asked whether compounds could interact with other kinases besides EphA2. Therefore, it was investigated their effect on other kinases as EGFR and VEGFR in cellular functional assays. By testing UniPR139, UniPR502, PCM505 at concentration inhibiting EphA2 activation, they resulted inactive against EGFR being unable to prevent the EGF induced EGFR phosphorylation (Fig.14A).

However, at the same concentration UniPR502 and PCM505 partially interfered with VEGF induced VEGFR2 activation (Fig.14B) without directly interfering with kinase activation when studied with LANCE assay at CEREP (data not shown). According to the literature, Eph-ephrin system is involved in the regulation of VEGFR signalling. It was demonstrated that Ephrin-B2 reverse signalling through PDZ interaction is a positive regulator of VEGFR2 and VEGFR3 activation [Sawamiphak et al. 2010; Wang et al. 2010]. Based on these findings, the inhibition of VEGFR2 phosphorylation induced by compounds could be a consequence of their antagonism on Eph receptors. Further studies are needing to clarify a putative inhibition of the compounds on VEGF-VEGFR2 binding.

Figure 14: A) Phosphorylation of EGFR in PC3. Cells were pretreated for 20 min with 0.3%DMSO or with the compounds at the indicated concentrations and then stimulated for 5 min with EGF 30ng/ml. Phospho-EGFR levels are relative to EGF + DMSO. B) Phosphorylation of VEGFR2 in HUVEC. Cells were pretreated for 20 min with 0.3% DMSO or with the compounds at the indicated concentrations and then stimulated for 10 min with VEGF 30ng/ml. Data are the means of at least 3 independent experiments±SD
*Phospho-VEGFR2 levels are relative to VEGF + DMSO. One-way ANOVA followed by Dunnet's post-test was performed to compare EGF+ DMSO to all the other columns (A) and VEGF+ DMSO control to all other columns (B) *p<0.05, ***p < 0.001.*



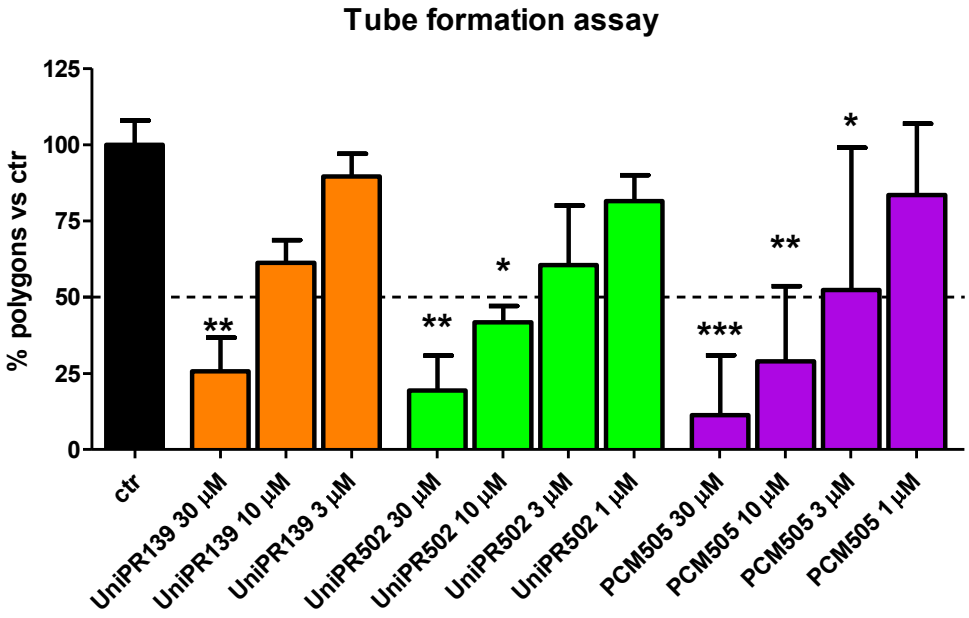
UniPR139 and PCM505 inhibit *in vitro* angiogenesis by EphA2 antagonism

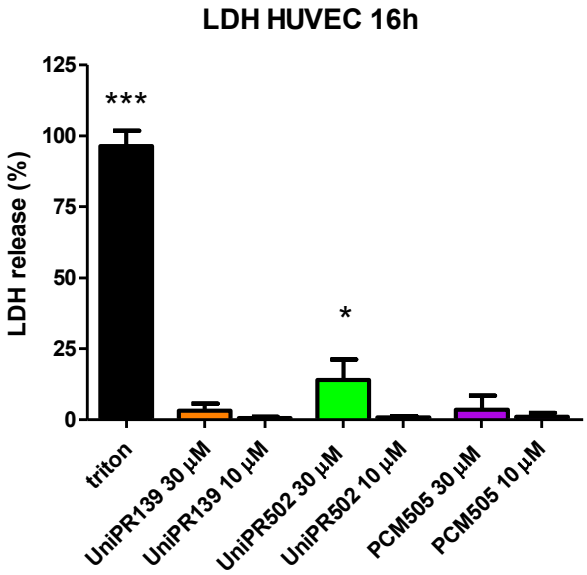
Angiogenesis is a crucial process for the growth and the spreading of tumor. Without it, tumor size would be restricted due to lack of nutrients, oxygen and clearance of metabolic wastes. As it is well known, Eph-ephrin system is involved in tumor angiogenesis and interference with this process could be another strategy for the disruption of tumor microenvironment. HUVECs have been used as a model for *in vitro* angiogenesis for some time. These cells consistently express Eph receptors [Pandey et al.1995] and they form a net of vessels when seeded onto a thin layer of Matrigel.

In order to evaluate *in vitro* anti-angiogenic properties of compounds, HUVECs were incubated for 15 hours with UniPR139, UniPR502 and PCM505. As shown from Fig. 15A, all compounds were able to inhibit the formation of vessels in a concentration-depend manner. At first sight, UniPR502 and PCM505 turned out the most anti-angiogenic compounds, showing an IC_{50} value around 6 and 3μM, respectively. However, when LDH assay was performed, by incubating compounds for the same time-span of tube formation assay, UniPR502 was the only one to cause a significant release of LDH at 30μM (Fig. 15B).

These results suggest as the block of angiogenesis by UniPR139 and PCM505 could be a consequence of Eph antagonism on endothelial cells, whereas the effect of UniPR502 on angiogenesis would be more referable to non-specific cytotoxicity.

*Figure 15: A) Tube formation assay in HUVE cells seeded on Matrigel. The number of polygons is relative to control (0.3% DMSO). B) Relative levels of LDH after 16h of incubation with compounds. Data are the means of at least 3 independent experiments ±SD. One-way ANOVA followed by Dunnet's post-test was performed to compare: control to all other columns (A); 0.3% DMSO control (0%, column not shown) to all other columns (B). *p < 0.05, **p < 0.01, ***p<0.001.*

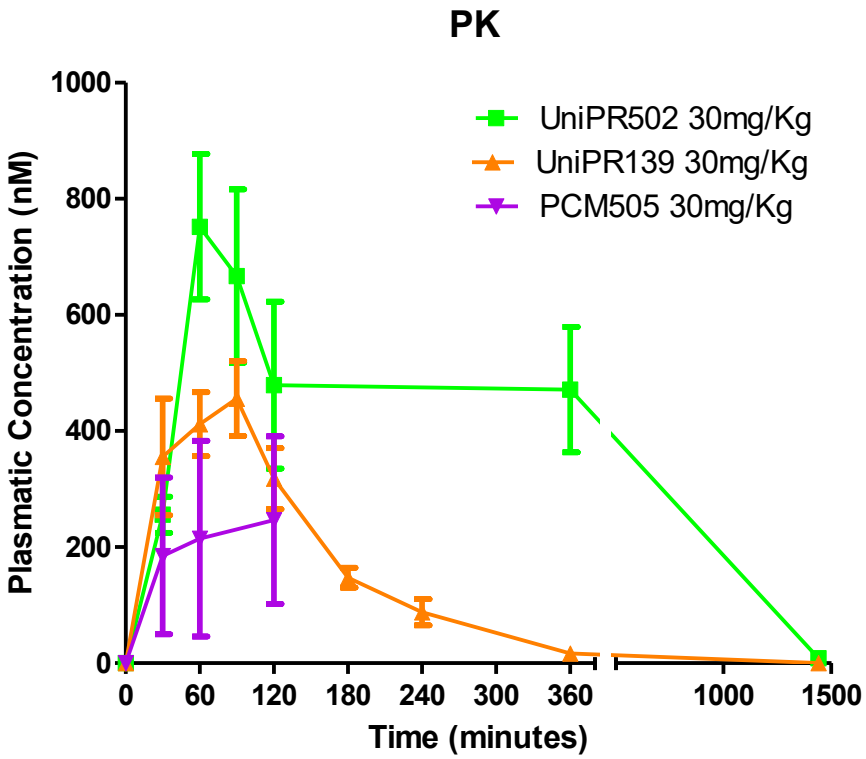




Bioavailability of UniPR139, UniPR502 and PCM505

The bioavailability of compounds was then evaluated on C57BL6/J male mice through oral administration of 30mg/kg UniPR502, UniPR139 or PCM505. Blood samples were collected via tail puncture, from 0 to 1440 minutes and the plasmatic concentration of compounds was quantified by HPLC-ESI-MS/MS thanks to Prof. Federica Vacondio and Dott. Francesca Ferlenghi of the Food and Drug department at University of Parma). UniPR502 was the most bioavailable compound with a C_{max} value of 790nM, $t_{1/2}$ of 177 min and AUC of 433 µM*min, whereas PCM505 was the lowest one, with a C_{max} of 250nM. UniPR139, instead, showed a C_{max} of 440nM, $t_{1/2}$ of 44minutes and AUC of 68 µM*min, values lower than UniPR502, but still acceptable to test it in animal models (Fig. 16). According to pharmacokinetic studies, UniPR502 and UniPR139 were the two compounds with better pharmacokinetic profile and considerably more bioavailable than their parental compound UniPR129 [Hassan-Mohamed et al. 2014]. For this reason, they were selected for *in vivo* studies

Figure 16 Pharmacokinetic: Plasma concentrations (nM) of the compounds over 1440 minutes time course after a single oral administration in mice (30 mg/kg) (n > 4).



Study of the effects of UniPR139 and UniPR502 in PC3 xenograft mice model

As reported in literature, EphA2 overexpression has been found in aggressive form of human prostate cancer compared to benign prostate tissues and it was related to oncogenic transformation and to the progress of disease [Zeng et al. 2003]. Moreover, EphA2 was correlated with vasculogenic mimicry *in vivo*, in fact, down-regulation of EphA2 hampered the formation of tubular structures in PC3 and DU145 cells [Wang et al. 2016]. Based on this scenario, targeting EphA2 receptor might reveal a potential therapeutic strategy and the use of EphA2 antagonist could be useful to block tumor growth in prostate cancer. Since our *in vitro* studies have showed as UniPR139 and UniPR502 were able to block the EphA2 forward signalling in PC3 cells, it was sought to test the compounds in an animal model of PC3-xenograft mice, to verify their ability to inhibit the tumorigenic activity.

UniPR139 and UniPR502 were daily orally administrated to BALB/c-nude mice at 30 mg/kg for 25 days. The anti-angiogenic drug sorafenib (2.5mg/kg/die/os) was used as standard treatment, in order to compare the activity of compounds with it.

Unfortunately, UniPR139 and UniPR502 failed to reduce the tumor growth *in vivo*. At the end of the treatment, tumors from each mouse were collected and weighted but no differences were observed by comparing controls group with UniPR139 andUniPR502 groups. Conversely, mice which were undergone to treatment with sorafenib had tumors with lower weight, indicative of the efficacy of drug (Fig.17).

Finally, it is worth note that toxicological studies showed as the treatment with UniPR139 and UniPR502 did not showed any major adverse effects (Table 6).

*Figure 17: Comparisons of tumour weight collected after 25 days of daily oral administration of UniPR139, UniPR502 and sorafenib. One-way ANOVA followed by Dunnet's post-test was performed to compare control group to all other treatment groups. **p < 0.01*

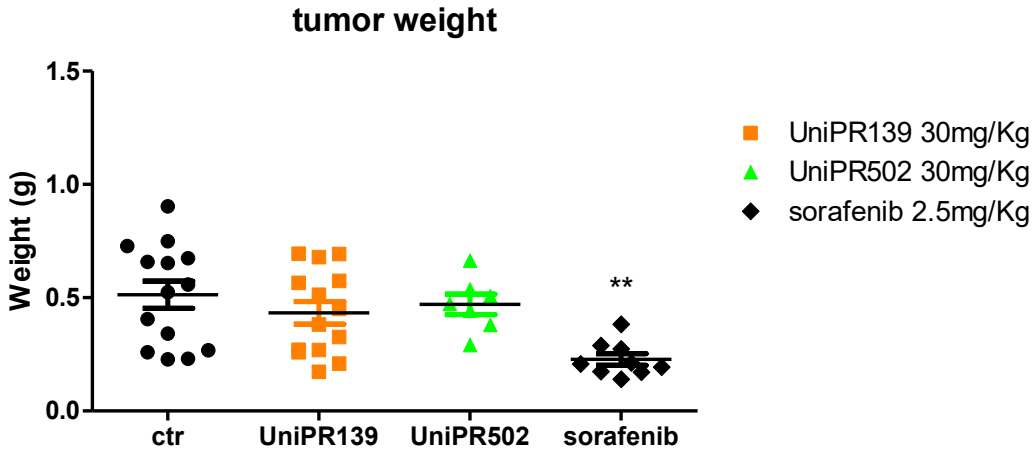


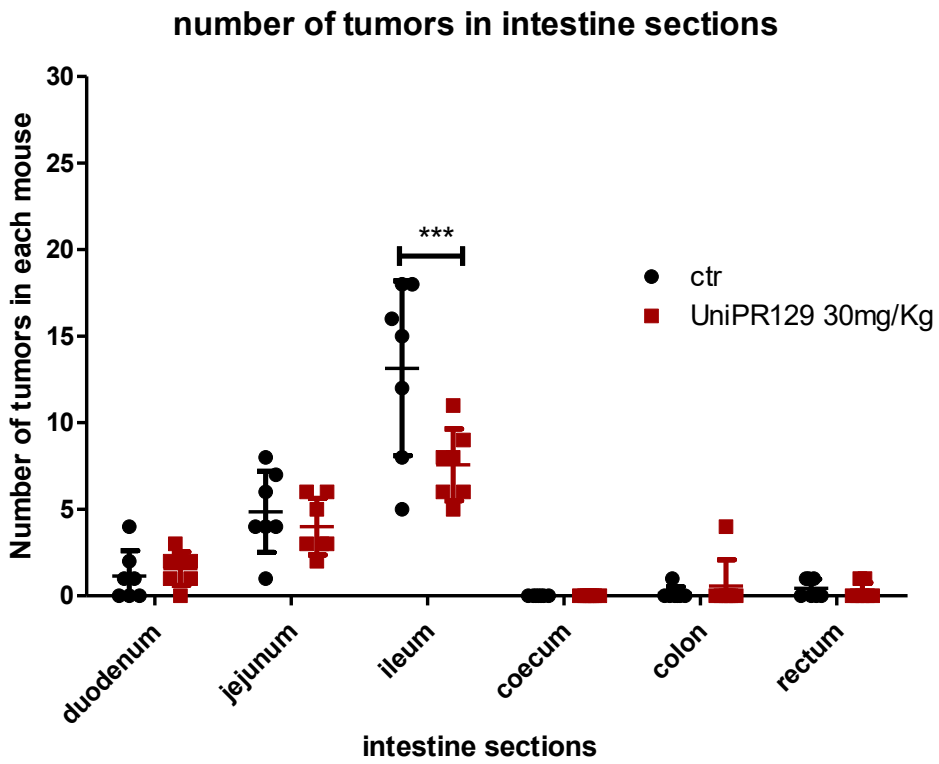
Table 6: Comparison of haematological parameters between control and UniPR139, UniPR502 group. One-Way ANOVA was used to compare the control to all other group for each parameter.

		CTR	UniPR139	UniPR502
		± SD	± SD	± SD
White cells	$10^3/\mu L$	7.13 ±1.63	6.90 ±0.79	9.13 ±1.65
Neutrophils	%	27.83 ±13.77	5.57 ±5.34	17.35 ±13.45
Lymphocytes	%	71.08 ±13.20	92.67 ±5.77	81.50 ±13.63
Monocytes	%	0.58 ±0.31	1.23 ±0.81	0.9 ±0.26
Eosinophils	%	0.35 ±0.19	0.33 ±0.06	0.33 ±0.36
Basophils	%	0.15 ±0.24	0.00 ±0.00	0.05 ±0.16
Red cells	$10^6/\mu L$	11.38 ±1.83	9.63 ±2.15	9.08 ±1.33
Haemoglobin	g/dL	15.55 ±1.54	15.47 ±2.20	15.68 ±1.53
Haematocrit	%	51.60 ±2.02	46.60 ±4.76	45.33 ±4.20
Mean cellular Volume	fL	51.40 ±1.24	51.97 ±0.25	51.33 ±1.23
Haemoglobin cell mean count	pG	15.18 ±0.74	14.53 ±0.61	15.70 ±1.2
Platelets	$10^3/\mu L$	1000 ±183	893 ±39	898±252
Mean platelet Volume	fL	5.43 ±0.38	5.47 ±0.72	5.08 ±0.26

Anti-tumor activity of UniPR19 in Apc^{Min}/J mice

UniPR129 was tested in Apc^{Min}/J mice, a mouse model of intestinal tumorigenesis. These animals are featured by a mutation gene thus they fast develop spontaneously multiply and large adenomas over intestinal tract. In Apc^{Min}/J mice, the distal portion of the small intestine is the site where the most of tumours occur, unlike the human where alteration of APC gene leads to the development of tumors mainly in the colon [Moser et al. 1995]. Even if UniPR129 turned out not oral bioavailable, because of extensive first pass hepatic metabolism (data not shown), the compound was tested in this model to verify if it could exert a local effect in the intestine. After 8 weeks of treatment, the number of adenomas in different sections of the intestine was counted by Prof. Anna Maria Cantoni of the Department of Veterinary Science at University of Parma and the treatment with 30mg/Kg of UniPR129 significantly reduced the formation of adenomas in the ileum (Fig. 18). No differences were observed in other tracts.

*Figure 18: Number of tumors developed from each mouse over sections of intestine in Apc^{Min}/J after 8 weeks of oral administration of UniPR129 30mg/Kg. Two-way ANOVA followed by Bonferroni's post-test was used to compare control to UniPR129 group. ***p < 0.001 N=7*



The diameter and the area of each single tumor in the ileum were measured and analyzed through ImageJ software. UniPR129 reduced tumor growth as appeared from the diameter and the area of tumors which were overall smaller than the control group (Fig.19 A,B). Moreover, subdividing tumor diameter in range of 0.5 mm and counting the number of tumors for each range, it emerged a significant difference between control and UniPR129 group for the tumor number with dimension ranging from 1 to 1.5mm (Fig.19C).

Finally, haematological and blood chemistry parameters were evaluated in order to rule out the possibility that UniPR129 could provoke toxic effects in mice. As observed from Table 7, the treatment with 30mg/Kg of UniPR129 was well tolerated from the animals and no meaningful changes were reported, confirming that UniPR129 reduced tumor growth in Apc^{Min}/J mice without adverse effects.

*Figure 19: Diameter (A) and area (B) of tumours collected in the Ileum in Apc^{Min}/J mice after 8 weeks of oral administration of UniPR129 30mg/Kg. Mann-Whitney test was performed to compare tumor diameter and area of control to UniPR129 group (*p < 0.05). C) Tumor dimensional distribution for control and UniPR129 group. Two-way ANOVA followed by Bonferroni's post-test was used to compare control group to UniPR129 group. *p < 0.05 *** p<0.001 N=7.*

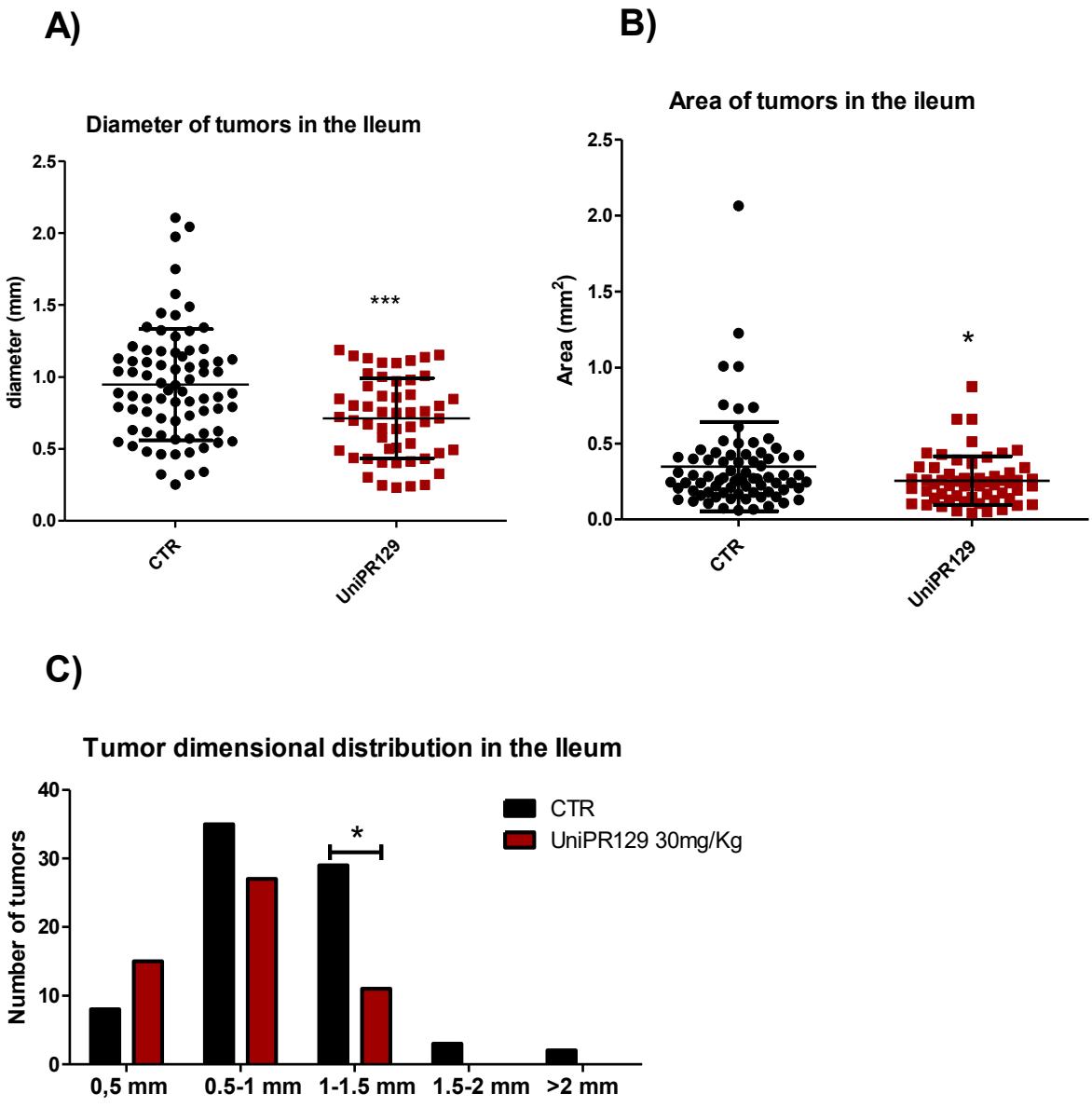


Table 7: Haematological and blood chemistry parameters. T-test was performed to compare control to UniPR129 group. SD= standard deviation. N=7

		CTR	UniPR129
		± SD	± SD
White cells	$10^3/\mu L$	5.53 ± 0.63	4,83 ± 0.26
Neutrophils	%	2.64 ± 0.58	6.20 ± 2.39
Lymphocytes	%	97.10 ± 0.59	93.29 ± 2.32
Monocytes	%	0.17 ± 0.02	0.43 ± 0.11
Eosinophils	%	0.07 ± 0.01	0.10 ± 0.03
Basophils	%	0.01 ± 0.014	0.00 ± 0.00
Red cells	$10^6/\mu L$	5.62 ± 0.39	5.01± 0.54
Haemoglobin	g/dL	9.70 ± 0.56	8.71 ± 0.81
Haematocrit	%	29.59 ± 1.53	26.69 ± 2.40
Mean cellular Volume	fL	52.99 ± 1.07	53.87 ± 0.99
Platelets	$10^3/\mu L$	1148± 92.49	1191 ±70.59
Mean platelet Volume	fL	5.01 ± 0.15	4.91±0.16
Creatinine	mg/dL	0.25 ± 0.02	0.22 ± 0.02
AST/GOT	UI	118.14 ± 21.09	108.86 ± 17.80
ALT	UI	30 ± 4.48	23.43 ± 1.42
Glucose	mg/dl	155.22 ± 10.57	161.10 ± 8.06
Triglycerides	mg/dl	156.3 ± 19.54	145.10 ± 14.58

DISCUSSION AND CONCLUSION

The Eph-ephrin system is implicated in tumorigenesis, although the exact mechanism of action has not been established, yet. Several studies have confirmed a deregulated function of ephrins and Eph receptors in different tumors and altered levels of these proteins have been associated with poor prognosis of patients, development of metastasis and tumor angiogenesis [Day et al. 2014; Taddei et al. 2011]. From this prospective, Eph receptors and their ligands seem promising targets for the development of new anti-cancer therapies. Small molecules which prevent the interaction between Eph-receptor and ephrin-ligand may find application for conditions where receptor activation contributes to the pathology.

The finding that the secondary bile acid, Lithocholic acid is a reversible and competitive antagonist of the EphA2 receptor, has prompted us to look for new more potent compounds with improved affinity [Giorgio et al. 2011]. The conjugation of the carboxylic group of Lithocholic acid [Incerti et al. 2017] with β-alanine and after the introduction of benzyl-imidazole in β-position of the amino acid, allowed on the one hand to markedly increase of the potency, shifting the Ki value from 49μM to 0.39μM as observed for UniPR129, and on the other hand [Incerti et al. 2017; Hassan-Mohamed et al. 2014] to acquire undesirable physio-chemical properties and fast liver metabolization.

For this reason, the structure of UniPR129 was explored and two series of compounds were synthetized by Prof. Alessio Lodola and Dott. Matteo Incerti, of Food and Drug Department through a combined structure-activity relationship analysis and Metadynamics.

UniPR139 was the most potent compound among the series of UniPR129 derivates where the indole ring of the amino acid was replaced with different aromatic groups. UniPR139 showed to bind the EphA2 receptor in a competitive and reversible manner with a K_i value of 950nM, almost 2.5-fold greater of the parental compound. As UniPR129, UniPR139 failed to discriminate the two subfamilies of Eph receptors, displaying only a minor preference for A-subfamily. This feature could turn out a benefit considering the redundancy of Eph/ephrin signalling due to the promiscuous interactions between Eph receptors and ephrins. It means that the activation of the same or various oncogenic pathways could be avoided by using non-selective small molecules as UniPR139, through the inhibition of different Eph receptors [Giorgio et al. 2018].

In cell-based assays UniPR139 acted as an EphA2 antagonist on PC3 cells, blocking the ephrin-A1 induced phosphorylation with an IC_{50} of 2μM, a value consistent with the one calculated in binding studies.

UniPR502 and PCM505 were, instead, the two best compounds of second series of UniPR129 derivates, bearing a carbamoyloxy group (UniPR502) or N-ethyl carbammates (PCM505) on 3α position. These two structural modifications allowed to get two reversible EphA2 binders, endowed with a potency similar to UniPR129. PCM505 showed to be inactive towards EphA1, EphA7, EphB1, EphB4 and EphB6 and to mainly bind the Eph A-subfamily receptors, especially EphA2. On the contrary UniPR502 was a pan-inhibitor, binding promiscuously EphAs and EphBs receptors.

The observation that carbamoyloxy functionality led to retain the inhibitory potency toward EphA2 receptors and to improve the physiochemical properties, prompted our collaborators (Prof. Alessio Lodola, Dr. Matteo Incerti and Dr Simonetta Russo) to extend these modifications on Lithocholic acid in order to evaluate how they could impact on the binding properties. Moreover, the introduction of carbamoyloxy group allowed to insert a point of chemical diversification into the Lithocholic acid structure, which is lacking functional groups that could provide further binding sites. Intriguingly, Lithocholic acid turned out more sensitive to substitution on 3α position than UniPR129, resulting in overall remarkable increase of the potency. The best result for this class was achieved with APO184, where a hexyl chain was inserted on Nitrogen. This compound competitively and reversibly bound the EphA2 receptor with an inhibitory potency in the low micromolar range. The Ki was 1.43μM, a value significantly smaller than Lithocholic acid but higher than UniPR129.

However, when UniPR139, UniPR502, PCM505 and APO184 were tested in functional studies, APO184 was the least effective to inhibit the activation of receptor, suggesting as the introduction of N-alkyl substituted carbamoyloxy group on Lithocholic acid improved the binding properties but it did not lead to a gain of inhibitory activity toward EphA2.

In order to assess the specificity of compounds, UniPR139, UniPR502 and PCM505 were tested as antagonist on EGFR and VEGFR2 and none of them interacted with EGFR, whereas UniPR502 and PCM505, partially reduced VEGF-induced VEGFR2 activation. This finding is not surprising as the interaction between Eph/ephrin system and VEGFR signalling is an event documented in literature. Wang et al. together with Sawamipack et al. provided compelling evidences that ephrin-B2 reverse signalling functions as a general modulator of the VEGFR pathway in endothelial cells during physiological and pathological angiogenesis, by facilitating

the internalization and the activation of VEGFR2 and VEGFR3 [Wang et al. 2010; Sawamiphak et al. 2010].

Moreover, it has been reported that also EphA2-ephrin-A1 signalling plays an important role for the VEGF-dependent angiogenesis and thus Eph/ephrin blocking agents may affect this process. [Cheng et al. 2002] With this in mind, it was investigated if UniPR139, UniPR502 and PCM505 could exert *in vitro* anti-angiogenic properties through the block of EphA2 activation. The three compounds inhibited the establishment of tubular structures on human umbilical vein endothelial cells, but a deeper analysis revealed as the effect exerted by UniPR502 was actually the result of non-specific cytotoxicity.

Finally, UniPR139 and UniPR502 showed to be bioavailable and suitable for oral administration in mice. This was a strikingly result if we consider that their parental compound, UniPR129, was barely detectable in the plasma. The achievement of oral bioavailability is one of the main sough-after features during the development of new molecules, which is fundamental for *in vivo* studies and for further clinical investigations. For this reason, this improvement represents an important step forward in the research of novel compounds targeting the Eph/ephrin system.

Based on these satisfying pharmacokinetic properties, the next step was to investigate the anti-tumor activity of compounds in PC3-xenografted nude mice. The findings that EphA2 receptor promoted proliferation of prostate cancer cells and that high level of EphA2 were observed in patients with malignant prostate cancer cells, make these proteins a potential target in oncology [Zeng et al. 2003]. Unfortunately, oral administration of UniPR139 and UniPR502 did not allow to achieve the expected results, in fact, compounds did not significantly prevent the tumor growth. One of the factors which could have negatively influenced the result of our study is the strong angiogenesis associated to this cancer. As documented in literature, tumor angiogenesis plays an important role in castration-resistant forms of prostate cancer, [Mukherji et al. 2013] and according to that the anti-angiogenic drug Sorafenib reduced significantly the tumor growth in our *in vivo* cancer model. However, although UniPR139 showed also anti-angiogenic activity in HUVEC cells due to the antagonism on EphA2, in our experimental setting this activity was not sufficient to inhibit the angiogenesis *in vivo* and thus the tumor growth.

Also, a macroscopic observation revealed as tumors harvested from control group displayed a more irregular shape than the one of UniPR139, UniPR502 groups. The treatment with compounds could have confined the tumor growth, avoiding the spreading of cells.

It should be considered also that the treatment with compounds started when tumors reached a volume of 200mm^3 in this experimental settings, but another approach could be the

administration of UniPR139, UniPR502 before the tumor occurrence in mice, in order to verify if they might prevent or arrest the tumor growth in these conditions.
Nevertheless, at that moment, further studies are needed to confirm this hypothesis.
Finally, the anti-tumor properties of UniPR129 were investigated on C57BL/6J-Apc^{Min}/J mice, a mouse model of intestinal tumorigenesis. This strain possesses a mutation such that leads to the spontaneously development of a large number of adenomas over whole intestinal tract and rarely survive beyond 120 days [Moser et al. 1995].
In normal intestine of Apc^{Min}/ J mice, EphA2 is mainly expressed in differentiated cells of the small intestine, such as in top of the crypts and in the villi, whereas it is absent or weakly expressed at the base of the crypts or in proliferating cells near stem cell niche. This diversification is lost once tumors are established in intestine of Apc^{Min}/ J mice and EphA2 becomes widely expressed, even in proliferating cells which are a major component of the tumor cell population. Moreover, Bogan et al. observed that EphA2 gene knockout Apc^{Min} / J mice developed fewer and smaller tumors in the small and large intestine [Bogan et al. 2009].
Based on these evidences, the aim of this experiment was to verify if UniPR129 could exert a local effect in the intestine, despite the lack of oral bioavailability. The compound reduced the number of adenomas in the ileum, as well as, it decreased the diameter and the area of adenomas in the same region. The restricted effect of UniPR129 in the ileum might be due to higher expression of EphA2 receptor in this region as reported in literature, and thus by the largest amount of available target for the compound.
In conclusion this study demonstrated as it was possible to get new Eph antagonists endowed with high inhibitory potency (UniPR502), *in vitro* anti-angiogenic activity and good pharmacokinetic properties (as UniPR139) by modifying the UniPR129 structure.
Although these compounds did not display the expected *in vivo* anti-tumor properties, it should be kept in mind that Eph receptors and ephrin ligands can exert a tumor promoter or tumor suppressor role depending of different factors, as cancer type, stage of disease and tumor microenvironment and EphA2 receptor is the landmark of this dichotomous effect. That suggests as the lack of anti-tumor effect observed for UniPR139 and UniPR502 could depend on the investigated cancer type. For example, UniPR1331 is another small molecule developed and synthetized by Dr. Castelli at University of Parma, resulting from the conjugation of cholenic acid with L-Tryptophan [Castelli et al. 2015]. As well as UniPR139, the compound is a pan Eph antagonist with anti-angiogenic properties, whereas contrary to UniPR139, it significantly reduced the tumor growth in xenograft and orthotopic glioblastoma mouse models [Festuccia et al. 2018]. However, the same compound was inactive when tested on PC3

xenograft (unpublished observations). Taken together, these data suggest that case by case study and tumor specific contest analysis should be considered during the pharmacological characterization of new anti-cancer agents targeting the Eph/ephrin system. Finally, further investigations of UniPR139 and UniPR502 should be carried out in other kinds of tumors in order to confirm or to exclude the anti-tumor activity of compounds.

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