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CICLO XXXVII

# Pharmacological Profiling of Novel PP-Inhibitors Derived from Cholenic Acid for Targeting Eph-Ephrin Signaling in Glioblastoma

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# GLIOBLASTOMA

Glioma is the type of cancer that originates in the brain. It may cover as much as 33% of the total brain tumours, arising from its glial cells, which are responsible for support and immunity within the nerve cells of the brain, thus including astrocytes, oligodendrocytes, and ependymal cells.

There is a basic division within gliomas, upon which some are with circumscribed growth patterns, whereas some have diffuse infiltration into the brain parenchyma[1].

Diffuse gliomas are classified according to WHO grading into four grades: WHO I, II, III and IV. Grade IV represents glioblastoma (GBM). It is also important to add that diffuse gliomas are common in adults than in children. The tumours are associated with a very short median survival phase-it only varies from 12 to 18 months for those graded IV[2].

Glioblastoma is a particularly dangerous form of brain tumour and the deadliest form of glioma because it does not respond to radio and chemotherapy. Treatment of GBM patients includes surgical resection of the tumour mass, subsequent radiotherapy treatment and administration of Temozolomide[3]. In the context of Glioblastoma, a distinction can be made between primary and recurrent forms of the disease. Primary GBM is characterized by the absence of any discernible clinical precursor at the time of presentation. In contrast, in recurrent GBM, there are molecular progresses in the continuum or an increase in grade of malignancy over time, and it usually arises from a lower-grade glioma[3,4].

Central to understanding the development and aggressiveness of GBM is to identify the cells responsible for tumour initiation. Before that, though, there must be a clear understanding of the development of glioma cells in the CNS. In developing CNS, NSCs can generate glial cells (astrocytes and oligodendrocytes) and neurons[5–7].

The detection of cell origin is one of the necessary components of classifying GBM subtypes and thought to be of consequence with respect to the path of tumour progression.

Single cell studies have revealed, in spite of inter-patient heterogeneity, tumour-responsive transcriptional programs tend to converge into a small number of well-organized states, which look similar to the canonical neuronal lineages found in the normal tissue[8–10].

The general belief is that Glioblastoma originates from three types of cells; neuronal stem cells, astrocytes originated in NSC and oligodendrocyte precursor cells[11].

There are several critical observations to this very dominant hypothesis. The first one is that between the GBM tissue and the normal cell-type of central nervous system, lay amazing similarities and expression and cell surface marker morphology. Second, genetic profiling analysis showed that there was a striking overlap between GBM and these normal CNS cells[11,12]. Finally, the authors provided experimental evidence from genetic modelling experiments in mice that different types of NSC cells, including NSC-derived astrocytes and OPCs, were competent to give rise to GBMs[13]. One can infer that glioblastoma cells are, to some extent, mirroring the same normal neurodevelopmental hierarchical organization (Fig.1).

Therefore, glioblastoma cells hatching from glial cells not only possess some characteristics of their parent cells but also manifest several abnormalities that set them apart very remarkably from new cells. These abnormalities are mutations in the regulators of gene expression that control cell cycle progression and DNA repair mechanisms, along with signalling pathways for growth and survival. Common genetic alterations seen in glioblastoma are amplifications of EGFR, PDGFR, amplifications, TP53, and PTEN mutations and IDH1, or CDK4 amplifications that can drive an AC-, OPC-, or NPC-like phenotype, respectively. In contrast, homozygous deletion of NF1 appears to skew glioblastoma cells toward a MES-like state[8,14,15].

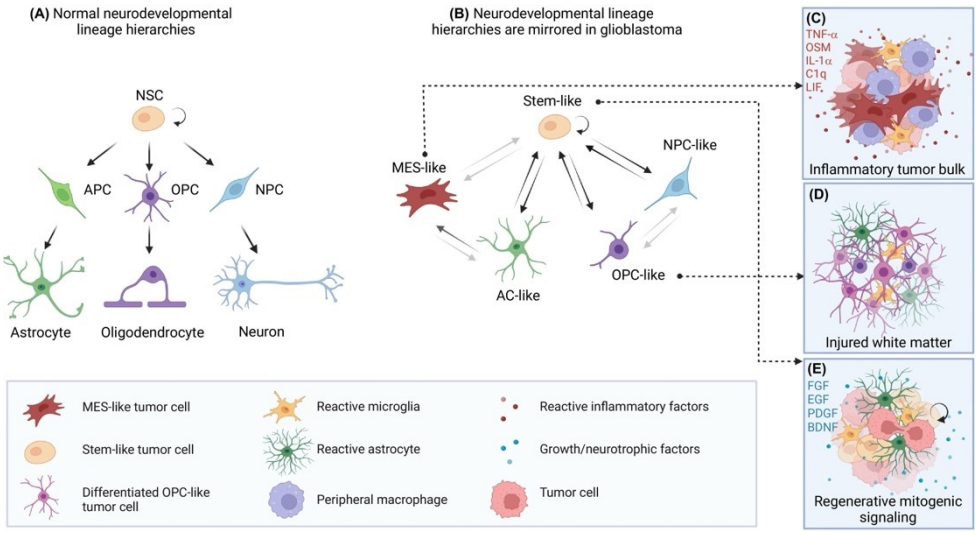



***Fig.1*** *Intra-tumoral transcriptional patterns sort into hierarchically organized states with similarities to normal neural lineages (L.J Brooks et al 2022).*

- **Glioblastoma and Stem Cells**

Literature indicates that in addition to these cell types, evidence can be found for another cell type within the different GBM patient samples: glioma stem-like cells. Small fractions of GSCs are self-renewing tumour cells of glioma origin that, despite their high tumorigenic potential, proliferate at a low rate. Moreover, they are resistant to chemotherapy and radiotherapy and lead to the recurrence of the tumour after treatment.

While already assessing the role of stem cells in glioblastoma, one can certainly say at this point that they have two properties: self-renewal and multiple lineages of differentiation. In combination, these two properties give shape to the concept of being a stem cell. More specifically, though, self-renewal is prominent alone, since its deregulation has been tightly associated with oncogenesis and malignancy[16,17].

Specific subpopulations of cells, referred to as "tumour-initiating" cells, have been identified in a variety of cancers, particularly those of the hematopoietic system, brain, and breast. Really extraordinary are these cells by their capacity for self-renewal and production of different cell types found within tumours, to reconstitute the proliferative ability of tumour cell populations[17–20]. Whereas it is plausible to suppose that cancer stem cells may result from abnormal mutations of normal stem cells, multiple studies suggest a different pathway. One of these pathways describes the pathway of mutation of progenitor cells as giving rise to cancer stem cells. Progenitor cells have large replicative potential, but they usually lack intrinsic self-renewal capabilities characteristic of stem cells. Therefore, the process by which progenitor cells are activated to cancer stem cells necessitates the acquisition of mutations that equip them with self-renewal potential[21–23].

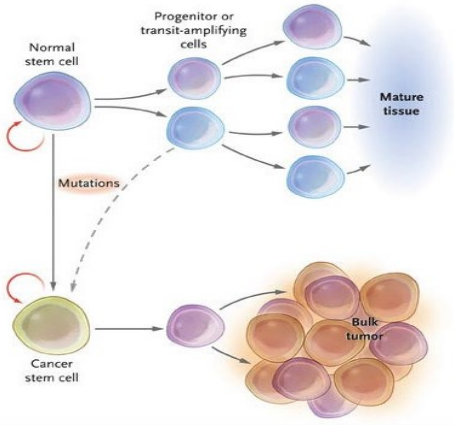



***Fig.2*** *Normal tissues are derived from central stem cells and then subsequently deliver progenitor and mature cell populations. Cancer stem cells arise through the mutagenesis*

*of normal stem or progenitor cells, allowing subsequent processes of proliferation and differentiation to produce the net tumour mass. (Craig T. Jordan et al.2016).*

Brain stem cells were first described by the group of Ignatova et al. [24], and their existence was then confirmed by several other laboratories[25–27]. Several Stemlike glioblastoma stem cells in glioblastoma were first described by Singh et al. as a subpopulation able to initiate a tumour i*n vivo*[20].

They possess the self-renewal capacity and produce all tumour cell types, thus conferring heterogeneity on the tumour. GSCs are a crucial component in a glioma's initiation, progression, and recurrence. They display what is called typical stem cell features or properties: the capacity for neurosphere formation after plating on a matrix in culture, the expression of some stem cell markers, and the formation of tumours following transplantation into animal models[27].

In the quest to unearth this classification, research indicates that the surface marker CD133 is an early identifier of the glioma-derived stem cells. The expression identifies GSCs and defines their self-renewal capacity, with a noted decrease in expression levels when the cells are undergoing differentiation[28]. This expression could clearly distinguish between the CD133+, which reflects the glioma stem cells from the CD133- cells, which do not represent the stem cells. Also, CD133+ cells are prone to producing neurons *in vitro* and to form brain tumours in animal models[20,29,30]. However, other markers like CD44, A2B5, CD90, transcription factor SOX2, enzymes ALDH1, L1CAM, KLF4, SALL4, GFAP has also been used to identify GSCs[31].

- **Resistance of Glioma Stem Cells**

Treatment resistance in GSCs is a redundant and multi-factorial event, which dramatically reduces the efficiency of treatments. Also, GSCs are ubiquitous within the tumour mass and can migrate through white matter tracts, thus making complete surgical removal impossible. White matter, composed by myelinated axons, acts as an essential route for glioblastoma development and propagation providing substrate for GSC migration[32–34].

Many studies and hypotheses have been pursued to clarify the mechanisms underlying this resistance.

The use of radiation therapy as reported before is one of the approaches in treating GBM. Ionizing radiation is effective against tumour cells due to it induces mutations in their DNA. Cells can activate specific DNA repair mechanisms and checkpoints in response to different types of damages. These processes thus enable the repairing of DNA damage so that cells can return to normal proliferation. Cellular response to DNA damage has been referred to as one of the main surviving mechanisms of tumour cells after radiotherapy[35,36]. Bao and his colleagues showed that subsets of CDs+ GCSs have resistance to ionizing radiation due to their more effective mechanism of repair. This mechanism implicates increased phosphorylation of CHK1, CHK2, a checkpoint kinase implicated in regulating cell cycle arrest and DNA repairing mechanisms. Hence, these cells undergo apoptosis less frequently than most of the tumour cells[32].

The most common therapy for Glioblastoma multiforme (GBM) is Temozolomide(TMZ), an orally administered alkylating agent and is known to methylate guanine at the O-6 position in DNA[37]. MGMT is a DNA repair protein that is crucial for removing the O-6 residue from methylated DNA. Its expression level dictates TMZ chemoresistance in GBM. MGMT expression varies within cases of GBM and was highly expressed in GSCs, which dramatically increased their resistance to TMZ[38–41]. Recent studies demonstrate that TMZ resistance was provided through an increase in MGMT expression via CD133+ GSCs[42].

MDR is the phenotype whereby cancer cells grow resistant to a wide spectrum of various, unrelated chemotherapeutic agents. This develops due to the induction of several mechanisms that include anti-apoptotic pathways, detoxification processes, reduced drug uptake, and activation of drug efflux by activation of transporters. So, of all gene families implicated in MDR, the ATP-binding cassette transporter family has been one of the well-studied families. These transporters extrude drugs from the cells, reducing the intracellular concentration of

drugs. Amongst such ABC transporters, MRP1 (ABCC1), MRP3 (ABCC3), and MRP4 (ABCC4) exhibit overexpression's in glioma cells. In addition, MRP1, MRP3, and MDR1 are also highly expressed in GSCs. CD133-positive GCSs have a much higher expression of MRP1 and MDR1, compared with their differentiated tumour cells. Moreover, etoposide and temozolomide increase the expression of MRP1 in CD133-positive U251 GSCs, possibly related to the intrinsic plasticity of cancer stem cells. Further, it has been reported that silencing of the anti-apoptotic protein resulted in inhibition of cell proliferation, downregulation of MRP1, and upregulation of MRP3 in GSCs. These findings imply that the major role executed by ABC transporters, especially MRP1, MRP3 and MDR1 in GSCs is to block drug entry. The different roles that each transporter plays, depending on the cellular context, therefore underscore the complexity of mechanisms leading to drug resistance in glioma [42].

Considering the aggressiveness of this tumour, research is committed to the development of new therapies and pharmacological strategies directed against Glioblastoma cells. One of the approaches has been the targeting of the interactions included in the Eph-ephrin system, as many components of this pathway are aberrantly expressed in GBM cells.

# EPH-EPHRIN SYSTEM

The Eph receptors represent the largest family of known tyrosine kinase receptors in mammals. The first Eph receptor was isolated in the late 1980s from erythropoietin-producing hepatocellular carcinoma cells. Eph receptors have been divided into two families: EphA and EphB. There are a total of sixteen Eph receptors that have been identified as ten of the sixteen are type A receptors while six are type B receptors[43]. The Eph receptors and their ligands, the ephrins, are transmembrane proteins that interact primarily through direct cell-cell contact. Physiologically, the Eph/ephrin complex stays core and central to most developmental processes, more particularly those concerned with brain development. It participates in critical events such as axonal guidance, vasculogenesis, and coordination of excitatory synaptic formations. The Eph/ephrin complex is involved more broadly in embryogenesis, providing the interconnecting material in developing tissues and controlling such basic processes as cellular adhesion, migration, and differentiation. Its effects are far-reaching to include such detailed developmental events as neural pattern formation in the auditory system and cortical dendrite growth and maturation. However, in adulthood, such an imbalance of the complex in various ways leads to the development of various pathologies, among which there are many types of cancer and malignant tumors[44–46].

The A and B receptors share striking structural homology, typified by:

- An extracellular N-terminal domain responsible for ephrin ligand recognition.
- A cysteine-rich domain.
- Two fibronectin type III domains.
- An intracellular juxta-membrane domain harbouring two phosphorylatable tyrosine residues.
- A tyrosine kinase domain comprising two phosphorylation sites.
- A sterile alpha motif (SAM) domain.
- A PDZ binding domain.

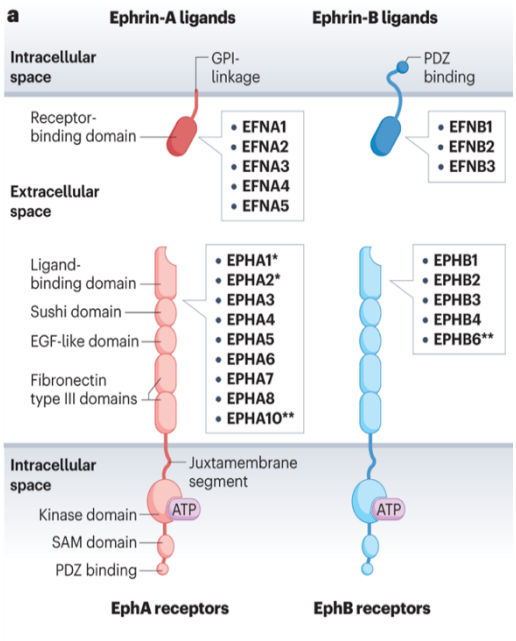



***Fig.3*** *Structure of Eph receptors and their ligands (Elena B.Pasquale 2024).*

- **Signal transduction**

The signal transduction of Eph/ephrin can manifests through various mechanisms: bidirectional signal transduction occurs through intercellular interaction, where one cell expresses the Eph receptor and the other the ephrin ligand, a process termed trans-activation.
It could be possible to activate the receptor through the generation of soluble ephrin ligand fragments by the activity of metalloprotease.
Furthermore, Eph receptor activation can occur independently of ligands. In this case, the interaction between Eph receptors and ephrin ligands *in cis* configurations takes place within the same cell entity.

- **Bidirectional signalling**

The bidirectional signalling transduction usually takes place after cell-to-cell contact between cells separately expressing the Eph receptor and ephrin ligand. The interaction triggers "FORWARD SIGNALING" through the Eph receptor-bearing cell and "REVERSE SIGNALING" through the ephrin ligand-expressing cell.
Concerning the mechanism of forward signalling, generally, Eph receptors dimerize upon contact with its ligand ephrin; afterwards, it undergoes tyrosine phosphorylation inclusive of serine residues present in their juxtamembrane part. This, therefore, allows the activation of the tyrosine kinase receptor for the generation of downstream activity.
Following the phosphorylation of Eph receptors, there is a consequent recruitment of diverse effector proteins:

- SH2 domain is found in proteins which become selectively associated with tyrosine motifs in the Juxtarmembrane and Kinase domains. These proteins, in turn act as key transducers in signalling cascades[47,48].
- The Rho GTPase family of proteins, which includes RhoA, Rac1, and Cdc42, have a very intricate role in the regulation of the cytoskeleton and influence morphological, adhesive, and motile cell phenotypes. Their general role in modulating these molecules is very important for various cellular processes[49].
- Modulation of the EphA receptors' kinase activity and subsequent binding to its Kinase domain exclusively in the nervous system utiliser the GEF ephexin. This molecular interaction plays a key role in regulating neuronal development and function[50].

In **reverse signalling**, there is a different signal transduction mechanism between type A and B ephrins.

Upon Eph/ephrin binding, a conformational change occurs in the intracellular domains of type B ephrin, exposing tyrosine residues in the C-terminal intracellular region. These residues can be phosphorylated by Src kinase, leading to create new binding sites for SH2 domains of other intracellular signalling proteins such as the adapter protein Grb4, involved in the axon pruning [49].

Moreover, proteins harbouring PDZ domains have been observed to interface with ephrin-B. Notably, the PDZ-RGS3 adapter serves to link Ephrin-B with G-protein receptors, thereby modulating neural cell migration and the self-renewal of neuronal precursors. Furthermore, interactions between ephrin-B and PDZ domain-containing proteins are implicated in critical biological processes such as angiogenesis and lymphangiogenesis, facilitating the internalization of vascular endothelial growth factor (VEGF) receptors [51–54].

With respect to ephrins-A, the lack of a cytoplasmic domain poses significant problems to understanding mechanisms underpinning their intracellular signalling. Studies in neuronal systems have revealed that p75 neurotrophin receptor can contribute to reverse signalling, but only in combination with other tyrosine kinase receptors like TrkB and Ret. This activity depends on these receptors acting as transmembrane binding partners for type A ephrins, thus allowing activation of reverse signalling pathways[49].

- **Signalling by metalloproteases**

Signalling mediated by the action of metalloproteases invariably relies on ligand dependency. Studies have clarified that certain membrane metalloproteases (MMP-1, -2, -9, -13) possess the capability to liberate soluble monomeric forms of ephrin ligands. These soluble ephrins, released into the extracellular environment by metalloproteases, exhibit the capacity to bind and activate Eph receptors: could be led to the shutdown of the signalling. *In vitro* investigations have highlighted that the soluble monomeric form of ephrin-A1 elicits down-regulation of EphA2. Additionally, pronounced alterations in tumour cell morphology have been noted, accompanied by attenuation of ERK phosphorylation and impairment of cellular migration and proliferation[55,56].

- ***CIS* signalling**

Another type of Eph/ephrin complex signalling is known as *cis* signalling. This type of signalling occurs when both the receptor and the ligand are expressed on the same cell.
This does not lead to activate signalling, but it seems to interfere with receptors activation. This has been hypothesized to provide an inhibitory signal that contributes to proliferation and tumour progression. Of interest, Eph receptors in tumour cells are overexpressed but exhibit low levels of phosphorylation at tyrosine residues, a phenomenon in part attributed to the blockade of trans-signalling mediated by *cis* interactions.
One distinguishing feature of *cis* signalling, as opposed to trans receptor activation, lies in the specific binding domains involved between the receptor and the ligand. While Eph-ephrin binding in trans predominantly involves the interaction between the G-H loop of ephrin and a specific binding pocket within the Eph receptor's ligand-binding domain, *cis* interactions entail the engagement of the Eph receptor's fibronectin type III domains with an ephrin domain distinct from the G-H loop [57–59].
Moreover, as reported before the co-expression of Eph receptors and ephrins on tumour cells, thereby engaging in *cis* interactions, can suppress the *trans* interactions that would otherwise activate the Eph receptors. Of particular interest is the finding that both ephrin-A3 and ephrin-B2 are capable of inhibiting EphA3 activation through *cis* interactions. This suggests that the two modes of *cis* and *trans* interaction do not exhibit identical receptor-ligand selectivity. Notably, ephrin-B2, despite not being a natural ligand of EphA3, can establish an interaction when expressed on the same cell[60].

- **Ligand independent signalling**

Another mechanism of activation of Eph receptors involves ligand-independent pathways. Ephrin-dependent signal pathways can induce differences in effect compared to Ephrin-dependent signal pathways, as EphA2 shows. This receptor is highly involved in various cancers. EphA2 overexpression can induce oncogenic transformation: tumour promotion[61,62].
In response to ligand binding, EphA2 activates typical artefact tyrosine kinase activity that dampens the RAS–extracellular signal-regulated kinase ERK and phosphoinositide 3-kinase–AKT pathways and negatively regulates integrin-mediated cell adhesion. Conversely, under unsupervised ligand circumstances, EphA2 becomes a substrate for several serine–threonine

kinases, including AKT, p90 ribosomal S6 kinase, and protein kinase A, which phosphorylate EphA2 at S897. This noncanonical signalling mechanism converts EphA2 from a tumour suppressor into an oncogenic protein[63].

Phosphorylation at S897 is crucial for regulating multiple malignant cell behaviours, including glioma cell infiltration *in vivo*, non-small cell lung cancer metastasis, chemotherapy resistance in ovarian cancer, and trans-endothelial invasion of bone marrow endothelium by prostate cancer cells[64,65].

Shi and colleagues demonstrated that, in the absence of ligand engagement, EphA2 receptors assemble into multimers via symmetric Head-Head (LBD-LBD and Sushi-Sushi) and asymmetric Head-Tail (LBD-FN2) interactions. This oligomerization maintains the kinase domains in the intracellular regions apart, thereby promoting ligand-independent noncanonical signalling through AKT-, RSK1-, and PKA-mediated phosphorylation at S897. Thus, noncanonical EphA2 signalling significantly contributes to malignant progression[66].

## Role of Eph-ephrin system in Glioblastoma

The genes encoding Eph receptors and their ligands, ephrins, are frequently observed to be overexpressed or exhibit distinct expression patterns in various human cancers; conversely, the Eph/ephrin system is found to be expressed at low levels in normal adult tissues[67]. Mounting evidence indicates that alterations in Eph/ephrin system expression or functionality are associated with the acquisition of tumorigenic properties, increased tumour growth, angiogenesis, and the development of metastases in various human tumours, including GBM[68,69].

Eph receptors are commonly expressed on migrating glioblastoma tumour cells, particularly at the outer margin of the tumour mass, thereby facilitating invasion into the brain parenchyma[69].

Both receptor and ligand members play diverse roles in glioblastoma pathogenesis.

- **EphAs and ephrin-As**

**EphA2 and ephrin-A1**

Notably, EphA2 expression in glioblastoma (GBM) is considerably higher than in normal brain tissue. This view is supported by the substantial increase of EphA2 mRNA levels in human GBM specimens noted above when compared to normal brain tissue. This big difference in expressions reinforces the potential of EphA2 as a molecular marker for GBM and further defines its role within the context of glioblastomas pathology[70].

EphA2 has been recognized as a pivotal factor in supporting tumour-propagating cells with stem-like features to maintain an undifferentiated state in human glioblastoma (GBM). This assertion is supported by experimental evidence showing that the suppression of EphA2 through siRNA knockdown in human GBM cells, as well as the induction of EphA2 downregulation using ephrinA1-Fc ligand, results in a decline in self-renewal capacity and the induction of differentiation *in vitro*[71].

In glioblastoma, EphA2 functions at the molecular level through a series of distinct mechanisms. These encompass the attenuation of Erk phosphorylation, modification in Akt interaction, downregulation of Sox, and modulation of stem cell invasiveness, as demonstrated by Miao and

colleagues. Taken together, these findings suggest that EphA2-mediated regulation of stemness and invasiveness represents a significant component of glioma pathophysiology[64,71].

Regarding the role of EphA2 in the regulation of cell migration: it inhibits this process upon ligand-dependent activation while stimulating it upon ligand-independent activation. Experimental investigations conducted with glioma stem-like cells (GSCs) have revealed that ligand-independent promotion of cell migration by EphA2 necessitates Akt-mediated phosphorylation of serine 897. In contrast, ligand-dependent activation of EphA2 inhibits both the Ras/ERK pathway and the PI3K/Akt pathway, thereby suppressing cell proliferation and inducing apoptosis. Notably, overexpression of EphA2 facilitates Akt activation, leading to the phosphorylation of EphA2 on serine 897, consequently transforming EphA2 from a tumour suppressor into a pro-invasive oncogenic protein[64].

- **EphA3 and ephrin-A5**

In the process of pathogenesis of GBM, EphA3 has emerged as a player coequal in importance to EphA2.

EphA3 has been overexpressed in most tumours, including those of the mesenchymal type of glioblastoma. Loss of EphA3 impaired the formation and aggregation of tumour spheroids in glioblastoma cells *in vitro*.

*In vitro* studies revealed that EphA3 expression downregulates the MAPK/ERK pathway, leading to a decrease in cellular differentiation. Overexpression of the EphA3 receptor thus enhances the proliferation of undifferentiated cells and promotes an increase in a tumour cell's self-renewal capacity. This was further supported through the utilization of ephrin-A5-Fc, an EphA3 receptor ligand that induces receptor internalization and degradation, and by the employment of shRNA against EphA3 to knock down its expression. These were further confirmed to inhibit the cell proliferation and increase cellular differentiation based on experimental models of ephrin-A5-Fc treatment and EphA3 silencing with shRNA lentiviral vectors. This resulted in a reduced expression of major markers for undifferentiated GBM cells, including CD15, CD133, and alpha6 integrin[72].

- **EphBs and ephrin-Bs**

In glioblastoma (GBM), research has consistently revealed an upregulation of all members of the ephrin-B ligand family, contrasted with the common downregulation of ephrin-A members in GBM cells. Although the precise functions of EphB/ephrin-B signalling in GBM are still under investigation, recent studies suggest that one of its key roles in this context is to facilitate migration and invasion within the tumour microenvironment[73].

**EphB2 and ephrin-B2/B3**

Overexpressed EphB2 has been reported in many types of cancers, including glioblastoma (GBM) [73]. EphB2 has been shown to actively promote the invasiveness of GBM cells by initiating the R-Ras signal and regulates integrin activity[74]. However, it has been demonstrated that EphB2 is a suppressor of tumorigenesis in colorectal and prostate cancers[75,76]. This twin role of EphB2, to act either as an oncoprotein or tumour suppressor, would seem to be defined by cellular context and the microenvironment within the cancer.

Up to date, for instance, ephrin-B2 and ephrin-B3 have been demonstrated to participate in the GBM invasion process by their action as ligands for EphB2, establishing a new mechanism of promoting glioma invasion through cell-cell contact directly[73]. Ephrin-B2 and ephrin-B3 activated EphB2 upon heterocellular contact, which in turn leads to the induction of invasion through the EphB2 forward signalling pathway. It has also been shown that the reverse signalling through ephrin-B2 and ephrin-B3 ligands regulates the invasion of glioma cells by acting on the activity of Rac1 GTPase[73]. More importantly, high expressions of ephrin-B2 have been significantly correlated with poor short-term survival in patients with malignant astrocytomas.

**EphB4 and ephrin-B2**

Among these, EphB4 and ephrin-B2 are the most studied in vasculature, particularly in malignant brain tumours. Evidence has shown such expression to be on the endothelial cells in such tumours. More recently, the function of ephrin-B2 reverse signalling has been described to be fundamental for internalization and activation of VEGFR-2, and thus to govern vessel sprouting in developmental angiogenesis[77,78].

## Eph-ephrin and Glioma Stem cells

Extensive research efforts have been devoted to unravelling the intricate role of Eph receptors in glioma stem cells, and accumulating evidence is pointing to this important involvement. While Eph receptors are expressed very broadly in adult stem cell niches and in a variety of cancers including gliomas, their functional relevance for the biology of CSCs has only recently been looked at. These receptors, found abundantly in both normal stem cells and CSCs, play a crucial role in tumour growth and metastasis[79,80].

Diverse members of the Eph receptor family have been implicated in different facets of tumour progression, including stemness, GSC differentiation, and angiogenesis[81].

Among these receptors, EphA2 has emerged as a central effector driving the infiltrative invasion of GSCs, thereby underscoring the high significance of Eph receptors for GSC biology[82]. In glioblastoma CSCs, EphA2 expression is strongly upregulated and, more importantly, expression directly correlates with CSC size and the tumour-initiating potential of glioblastoma CSCs. Indeed, EphA2 signalling was demonstrated to be critically involved in the activation of CSC self-renewal, wherein downregulation of EphA2 by ephrin-A1 almost abrogated CSC self-renewal *in vitro* and *in vivo* tumorigenicity

The downregulation of the EphA2 receptor in glioma stem cells results in a significant decrease in their self-renewal capacity. This decrease in tumorigenicity is caused by a loss in the expression of stem cell genes and an increase in the expression of differentiation marker genes. It has also been seen that marker, such as CD133, CD15, SOX2, and Bmi1, reduce. The more these markers are expressed, the more the malignancy of the tumour and the undifferentiated phenotype of the cells. These findings, facilitated by knockdown of EphA2 by lentiviral vectors expressing shRNA directed against EPHA2, provide compelling and robust functional evidence of EphA2's critical role in cancer stem cells[83].

It has been shown further through other studies that EphA3 supports the growth of an undifferentiated and self-renewing population of tumour cells through modulation of the intracellular MAPK signalling pathway. Gene silencing of EphA3 expression has been shown to result in partial differentiation and reduced cell proliferation. Moreover, low EphA3 cell surface expression is correlated with attenuated cell growth; this suggests that the shift in cellular equilibrium from highly proliferative and undifferentiated to less tumorigenic and more differentiated cells with slow proliferation rates results from a decrease in EphA3 levels. Notably, EphA3 presence is essential for the functionality of tumour-propagating cells, as

receptor silencing diminishes their tumorigenic potential. EphA3 expression also co-localizes with certain malignancy markers such as Nestin. Similarly to EphA2, loss of EphA3 expression correlates with enhanced cellular differentiation and reduced expression of markers including Nestin, CD133, and integrin alpha6[72].

This role of ephrin-B2 has been further explored in glioma stem cells, where it was similarly demonstrated that overexpression of ephrin-B2 in tumour cells eventually resulted in the downmodulation of its receptor, EphB4. In consequence, confinement mechanisms mediated by vascular ephrin-B2 were overcome, and GSCs could move unrestrictedly inside capillaries. Furthermore, overexpression of ephrin-B2 facilitates cell proliferation independently of their interaction with blood vessels. This proliferation is enhanced by "reverse signalling," which activates RhoA-dependent cytokinesis. Thus, ephrin-B2 plays a central role in vascular invasion and the development of metastasis. Notably, the effects of proliferation and cell evasion are mediated by different signalling pathways of the ligand-receptor complex. GSCs' evasion from endothelial compartmentalization relies on continuous activation of Eph receptor "forward signalling" induced by high levels of ephrin-B2 through homotypic cell-cell interactions within the tumour cell population. This desensitizes the receptors from further activation by vascular ephrins, thereby neutralizing endothelial ephrin-B2 repulsion and allowing unimpeded perivascular migration. In contrast, the effects of ephrin-B2 on proliferation depend on "reverse signalling" and are independent of homotypic cell-cell contact[84].

# PHARMACOLOGICAL TOOLS

The involvement of the Eph-ephrin system in glioblastoma pathogenesis has become increasingly clear. Consequently, various research teams across the world are currently working and developing new strategies to target this system for the treatment of glioblastoma.

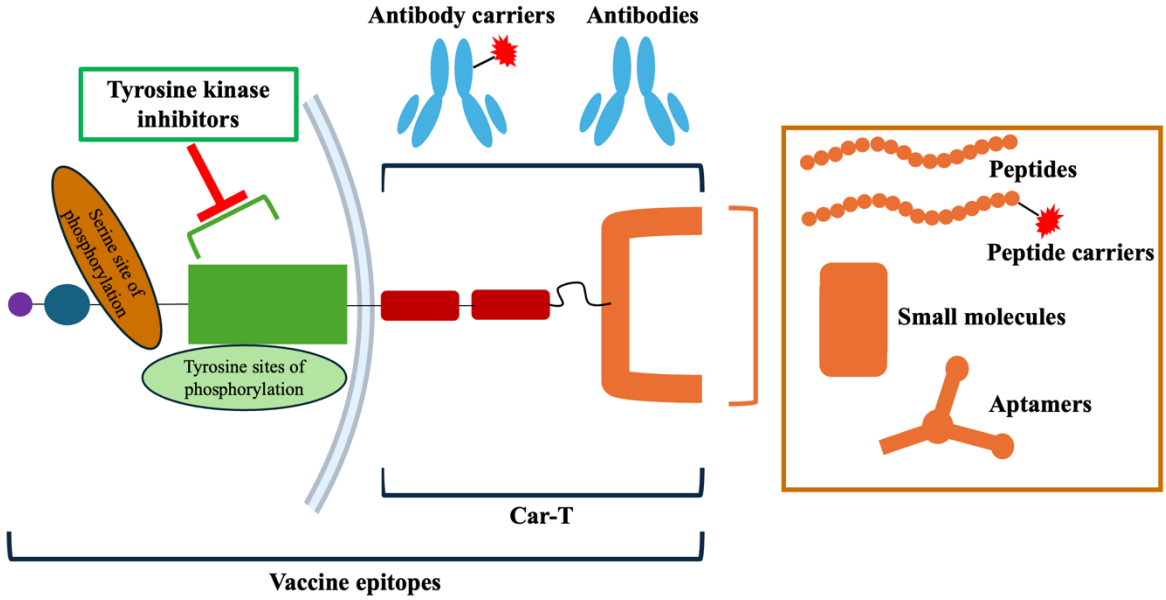



***Fig.4*** *Pharmacological tools developed on Eph receptors (C.Giorgio et al.2024).*

Here are detailed approaches and strategies for targeting Eph receptors:

- **<u>Tyrosine kinase inhibitors</u>**

As a result of the fact that Eph receptors are the largest family representing tyrosine kinase receptors, considerable efforts have been made to determine whether tyrosine kinase inhibitors would be effective in treating glioblastoma. Dasatinib was one of these potent tyrosine kinase inhibitors that is approved for treating chronic myeloid leukaemia and known to inhibit a multitude of kinases such as EphA2[85].

Different studies have been done to determine whether Dasatinib can directly inhibit the tyrosine kinase activity of EphA2 receptors. Cells overexpressing EphA2 were taken for experiments, and a reduction in receptor autophosphorylation was reported upon the administration of Dasatinib. Due to EphA2's implication in glioblastoma, Dasatinib was tested on therapy-

resistant GSCs. The findings indicate that Dasatinib effectively inhibits the migration of GSC cells but does not affect their proliferation[86,87].

The use of Dasatinib is limited by its very low selectivity for Eph receptors, as it inhibits a wide range of kinases.

While many tyrosine kinase inhibitors have been developed for a range of cancers, despite the recent interest in the role of EPHA2 in cancer progression, there is still a surprising lack of inhibitor chemical matter reported to target the kinase domain of EPHA2. While screening campaigns of compound libraries revealed micromolar binders, such as catechol and quinazoline derivatives,[88], more recently, nanomolar inhibitors have been developed with some success, including GLPG1790 and ALW-II41-27. These findings are thus oriented toward, if already showing, potentially more efficient therapies directed at EPHA2 in cancer treatment.

GLPG1790 is a small molecule inhibitor of different Eph receptor kinases. GLPG1790 was screened against a set of preclinical GBM models *in vitro* and *in vivo* and showed effective inhibition of ephrin-A1-mediated phosphorylation at Tyr588 and Ser897, resulting in complete abrogation of EphA2 receptor signalling. Similarly, this compound blocks ephrin B2-mediated EphA3 and EphB4 tyrosine phosphorylation. These actions result in reduced tumour growth *in vivo* and a downregulation of mesenchymal markers such as CD44, Sox2, and nestin[81].

ALW-II-41-27 is a type II small molecule inhibitor that effectively targets both the ATP-binding region and an allosteric site near the "DFG" motif in EphA2, thus inhibiting its kinase activity. It has been reported that ALW-II-41-27 inhibits EphA2 kinase activity with an $IC_{50}$ of 11 nM in NSCLC cells and that it suppresses *in vitro* cell survival and proliferation, inducing apoptosis in NSCLC cells. However, in an NSCLC xenograft model *in vivo*, rather suboptimal pharmacokinetics and limited oral bioavailability were revealed for ALW-II-41-27 when administered. Intraperitoneal injections of ALW-II-41-27 in mice inhibited the growth of set up tumours significantly. Further *in vivo* studies confirmed the selectivity of ALW-II-41-27 toward EphA2, despite its interaction with several intracellular kinases *in vitro*, such as Abelson, p38 mitogen-activated protein kinase, and multiple SRC-family kinases[89,90].

- **Antibodies and cytotoxic agents**

Targeting of EphA3 by a monoclonal antibody to attain extremely high specificity in action against GBM cells, conjugated with a Lutetium radioisotope has been developed. The evaluated antibody in an orthotopic murine model of GBM whereby U251 or BAH1 cells had been implanted in immunocompromised mice. Administrations of Lu-IIIA4 mAb at the highest treatment doses led to the complete regression of the tumour within 9 weeks and no case of regrowth of the tumour was observed even after 17 weeks. Histological analysis showed that Lu-IIIA4 mAb induced apoptosis of the tumour cells, likely mediated through the radioactive lutetium isotope. This treatment caused no apparent side effects, probably due to the very low expression of EphA3 in normal tissues[72].

As previously discussed, EphA2 and EphA3 play crucial roles in maintaining tumorigenic potential and the survival of GSCs. Consequently, a bispecific antibody has been engineered to target both EphA2 and EphA3.*In vitro* studies with this antibody in human GBM cells show a constitutive loss of EphA2 from the outside cellular environment and a 50% decrease in EphA3 expression. Such downregulation of two Eph receptors results in decreased AKT and ERK ½ activity. This treatment regime decreases the clonogenic and proliferative potential of tumour cells. Importantly, such decreases are independent of changes at the level in the cell cycle as well as activation of apoptotic pathways[83].

Another proposed intervention strategy is the conjugation of the exotoxin A produced by Pseudomonas aeruginosa with ephrinA1-Fc. This conjugation specifically aims to achieve action specificity towards glioblastoma by exploiting the overexpression of EphA2 while preserving surrounding healthy tissues. The exotoxin A variant (PE38QQR), which has been purified to eliminate toxicity to the organism, functions by blocking protein synthesis through targeting the protein elongation factor upon internalization. Additionally, *in vitro* studies demonstrated that ephrinA1-PE38QQR inhibits EphA2-overexpressing GBM cells[91].

Sharma and co-workers have characterized a QUAD 3.0 multivalent protein carrier in their work. The human IgG scaffold, with a fusion of IL-13, contains a terminal domain that has shown cysteine residue to be able to conjugate other proteins or conjugate chemotherapeutic agents. Specifically, the antibody was conjugated with recombinant ephrin-A5 to target EphA3, EphA2, EphB2, and IL-13RA2 receptors. Subsequently, the complex was conjugated with derivatives of Doxorubicin, an anthracycline known to inhibit nucleic acid synthesis and cellular mitosis by binding to cellular DNA[92].

Another example is the antibodies developed by Dowdy Jackso and colleagues to bring about an immune response and inhibit the growth of cancer cells. This research team produced an immunoconjugate via linking an EphA2 antibody to a cytotoxic agent, specifically an auristatin analogue. Recent findings indicate that the conjugation of an anti-EphA2 antibody to an auristatin analogue has proven to be very effective for the treatment of tumours that overexpress the EphA2 receptor. This approach led to the creation of an immunoconjugate composed of an anti-EphA2 monoclonal antibody (1C1), acquired by AstraZeneca, linked to maleimidocaproyl-monomethyl auristatin phenylalanine (mcMMAF). In its unconjugated form, the antibody exhibits no antitumor effects. To enhance its efficacy, it has been engineered to deliver highly toxic chemotherapy agents directly to EphA2-expressing cancer cells. One such agent is auristatin, a microtubule inhibitor that binds to the vinca alkaloid-binding domain and has shown potent anticancer effects in both preclinical and clinical studies. Upon internalization of the antibody, named MEDI-547, the cysteine-mcMMAF (cys-mcMMAF) is released from 1C1 through lysosomal degradation of the antibody component. This release leads to cell cycle arrest at the G2–M phase transition, microtubule disruption, and apoptotic cell death. The clinical trials of Medi-547 (NCT00796055) is discontinued discontinued because of bleeding and coagulation toxicity during Phase 1[93].

Toshinori Agatsuma research group has developed DS-8895a, an afucosylated humanized monoclonal antibody (mAb) targeting EPHA2, for cancer treatment. It has been shown to recognize the juxtamembrane region of EPHA2's extracellular domain, probably resulting in the ability to bind both full-length and truncated forms of EPHA2. These are anchored to cell membranes and produced by post-translational cleavage in tumours. In this regard, *in vitro* studies showed DS-8895a to significantly enhance antibody-dependent cellular cytotoxicity. It also inhibited the *in vivo* growth of human EPHA2-positive breast cancer xenografts established with MDA-MB-231 cells and human gastric cancer xenografts established with SNU-16 cells. DS-8895a: Phase 1 Study of  in Subjects With Advanced Solid Tumours - NCT02004717[94].

- **Peptides**

Peptides can bind the ligand-binding domain of the Eph receptor with high affinity, are relatively non-toxic, and do not need to cross the plasma membrane in order to engage the receptor[95].

In particular, peptides such as YSA and SWL have been developed that specifically bind to EphA2, modulate its activity, and reduce proliferation, reducing invasiveness of EphA2-overexpressing tumour cells. Pharmacokinetically, YSA and SWL are stable in cell culture medium but not in plasma, where they are rapidly degraded by aminopeptidases. This degradation adversely affects their ability to reach effective plasma concentrations [95–97].

For the EphB2 receptor, a peptide termed SNEW has been synthesized. SNEW binds to the EphB2 receptor with high selectivity and suppresses its signalling, functioning as a receptor antagonist [95].

Another series of peptides have been synthesized by Professor Pellecchia's research group. These peptides, named 135H11 and 135H12, exhibit high affinity for binding EphA2. *In vitro* studies on pancreatic cancer cells have demonstrated that the dimeric form, 135H12, is more potent in degrading the EphA2 receptor and dephosphorylating serine 897 on EphA2 compared to its monomeric counterpart[98].

Finally, a bicycle peptide, named BT5528, conjugated to the auristatin derivative maleimidocaproyl-monomethyl auristatin E has been shown to inhibit tumour growth in mouse models of fibrosarcoma and prostate cancer, as well as in a patient-derived xenograft model of non-small cell lung cancer. Furthermore, another invention by this research group involves linking an EphA2-targeting bicycle peptide with BCY12491. This agent effectively induced a CD8+ T cell response against the tumour mass in a syngeneic colon cancer xenograft mouse model that is transgenic for human CD137 [99]. A phase I/II multicentre, open-label study is underway for BT5528 as a single agent and in combination with anti-PD1 antibody Nivolumab in patients with EphA2 receptor-expressing solid tumours. This study evaluates the safety and tolerability of BT5528 and determines a recommended dose for further evaluation. The clinical activity will be assessed in phase II. Results are not reported at this moment.

- **Small molecules**

An alternative strategy for targeting Eph receptors involves the use of small molecule agonists or antagonists that bind to the ephrin-binding pocket of these receptors.

Among these we can find some agonist of EphA2 developed by the Case western reserve university in the research group of Wang B. These molecules were designed and synthetized from Doxazosin: an α1 antagonist showing a weak agonist effect on EphA2. These new derivates were shown an ability to internalize the EphA2 receptor and also to inhibit glioblastoma (GBM) cancer cell lines in *in vitro* experiments[100].

In order to develop protein-protein inhibitors capable of disrupting EphA2-ephrin-A1 interaction a screening of different compounds (HTS) based on ELISA-type binding tests was conducted at the Department of Pharmacy of the University of Parma. It allowed the identification of the lithocholic acid (LCA), a secondary biliary acid, capable of acting as a competitive inhibitor of EphA2-ephrinA1 binding. LCA is able to act at micromolar concentrations as a competitive and reversible inhibitor of the EphA2 receptor and its ephrin-A1 ligand [101].

The discovery of lithocholic acid as a reversible, competitive antagonist of the Eph-ephrin system marked the beginning of an extensive research campaign aimed at identifying novel molecules with enhanced affinity and potency. Utilizing the structure of lithocholic acid as a scaffold, structure-activity relationship (SAR) analyses facilitated the synthesis and evaluation of new derivatives for their efficacy in disrupting the EphA2–ephrin-A1 interaction.

Initially, amino acid-conjugated lithocholic acid derivatives had been designed, which were showing micromolar potency against inhibition of the EphA2-ephrin-A1 interaction. Two compounds in particular, N-(3α-hydroxy-5β-cholan-24-oyl)-L-tryptophan and N-(3α-hydroxy-5β-cholan-24-oyl)-Lβ-homotryptophan, emerged as top hits. These compounds displayed potent inhibitory activity. However, poor physicochemical properties and low selectivity limited thus far the practical applications as Eph-ephrin antagonists to be restricted to *in vitro* studies. Furthermore, such compounds did not achieve therapeutic plasma levels relevant for *in vivo* experiments upon oral administration[101].

Due to these drawbacks or limitations, a collaborative structure- and ligand-based virtual screening approach has been applied on a library of commercially available compounds to hopefully identify more potent molecules. This strategy was meant to identify lipophilic carboxylic acids as analogues of lithocholic acid. Among the selected compounds, 3β-hydroxy-

Δ5-cholenic acid was identified as a micromolar inhibitor of the EphA2-ephrin-A1 interaction, with an $IC_{50}$ value of 4.40 μM[102].

Notably, a molecule named UniPR1331 has been identified as a promising antagonist of the Eph-ephrin system. UniPR1331 has been reported to interfere *in vitro* with the EphA2 receptor–ephrin-A1 interaction at low micromolar concentrations, leading to angiogenesis inhibition. Further, oral bioavailability of UniPR1331 in mice without off-target interaction has been identified. *In vivo*, in the case of immunodeficient mice carrying xenografted U87MG and U251MG cell lines, UniPR1331 displayed a marked reduction in tumour growth. Biochemical and histological analyses showed that reduction in the growth of tumours with these compounds was mainly caused by a high reduction in blood vessel formation, vasculomimicry inhibition, and induction of apoptosis. [103].

- **CAR-T**

CAR T cell therapy stands supreme as a cancer immunotherapy whereby a patient's T cells are genetically modified to become more potent at finding and destroying cancerous cells in the body. It is an FDA-approved treatment for leukaemia, lymphoma, and myeloma. Building on the success of adoptive T cell therapies, such as chimeric antigen receptor T-cell therapy in various cancers, several studies have been investigated using genetically modified T cells expressing the chimeric antigen receptor that targets EphA2 in various tumours. Specifically, Zhong X. filed patent applications relating to T cells engineered with chimeric antigen receptors for different epitopes of EphA2. Ex vivo-activated and -expanded genetically modified T cells have shown enhanced survival in murine models bearing syngeneic GBM104 after activation *in vitro* against EphA2-positive glioblastoma cells[104].

- **Epitopes**

The research group of Storkus at the University of Pittsburgh has developed protein fragments containing EphA2 T-cell epitopes for vaccination using EphA2 peptide-pulsed dendritic cells (Eph-DC)-based vaccines. These innovations have shown effectiveness in preventing both the initiation and growth of tumour masses in diverse mouse models, fostering a specific cytotoxic T-cell response. This approach was further evaluated in clinical trials (NCT01876212) involving patients with metastatic melanoma who received treatment alongside the Dasatinib[105].

# RESEARCH AIM

There are mounting evidence that the Eph-ephrin system is involved in several pathological processes, especially in cancer. Altered expression of this system has been observed in many tumours such as breast, colon, liver, prostate, and glioblastoma. Eph receptors and their ligands have been related with malignant progression, tumour angiogenesis, metastasis, propagation and maintenance of tumour stem cell. Looking at this evidence, it is reasonable 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 contradictory 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.

As noted in the introduction, UniPR1331, an Eph receptors pan-inhibitor, has demonstrated activity in disrupting the EphA2-ephrin-A1 interaction and has shown efficacy in reducing tumour growth in an *in vivo* model[103].

However, it is important to remember that the Eph-ephrin system is a redundant system, in which almost every ephrin can bind to multiple Eph kinases[67]. Thus, the silencing of one single ephrin-or Eph kinase-could easily be compensated by other members of the family. Thereby, this redundancy makes it difficult to assess to what extent the two subfamilies, A and B, each contribute to the various processes that support tumor growth.

Thus, my research might be the starting point for a more extended investigation that will contribute to explaining the role of these two subfamilies in detail in different tumorigenic processes. Indeed, the treatment with selective molecules active on either A or B subfamily could clarify the role played by the two subfamilies in modulating the different tumorigenic processes of glioblastoma.

Based on that and thanks to the computational analyses developed by Professor A. Lodola's research group, the synthesis of UniPR1447 was achieved. UniPR1447 is a beta-homologue of UniPR1331. UniPR1447 (1), specifically, is the L-β-homotryptophan conjugate of 3-β-hydroxy-Δ5-cholenic acid. This compound interacts with the EphA2 receptor by accommodating its rigid core within a hydrophobic channel, with its carboxylic group forming

a salt bridge with Arg103 and its indole ring positioned in an accessory pocket adjacent to Met73. The presence of this accessory pocket is a distinctive feature of the ligand-binding domain (LBD) of EphA2, absent in EphB2.

Exploiting this unique characteristic, a N-sulfonylphenyl substituent was introduced at the indole nitrogen atom of UniPR1447(1). This modification led to the synthesis of compounds UniPR1449 (2). These compounds exhibited significant affinity for the EphA2 receptor while demonstrating no interaction with different EphB receptors at concentrations up to 30 μM. The introduction of the bulky N-sulfonylphenyl substituent conferred a degree of selectivity for EphA2, underscoring the potential for developing EphA2-specific antagonists.

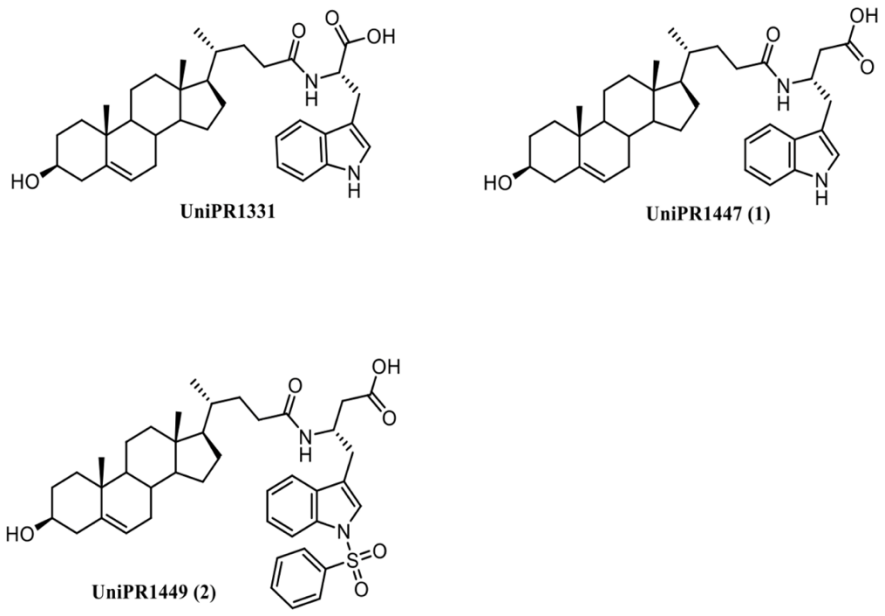



***Fig.5*** *Graphical representation of compounds obtained from UniPR1331.*

UniPR1331 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 through functional assays performed on cells. Finally, compounds were tested in pharmacokinetic studies.

# MATERIAL AND METHODS

- **Reagents**

All culture media and supplements were bought from Euroclone (Milano, Italy).
Recombinant proteins and antibodies were from R&D System (Minneapolis, Minnesota). Leupeptin, aprotinin, NP40, tween20, BSA, and salts for solution were from ITW Reagents (Chicago, Illinois); EDTA and sodium orthovanadate were from Merk (Darmstadt, Germany)

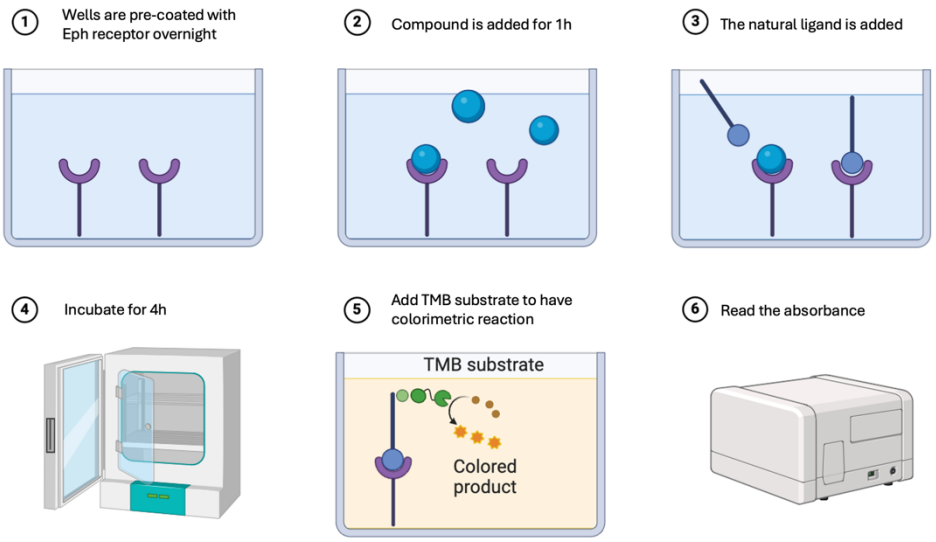
- **Cell cultures**

U251 (human glioblastoma cells) are cultured in EMEM supplemented with 10% FCS, 1% non-essential amino acids, 1% sodium pyruvate, and 1% antibiotic solution. The cell line is maintained in an incubator with a humidified atmosphere consisting of 95% air and 5% CO2 at a temperature of 37°C.
L-11: Patient-derived GCGR cell line was provided by the glioma cellular genetics resource: (www.gcgr.org.uk). It was cultured adherently in serum-free GSC media (DMEM/F-12 (Life Technologies) N2 (1/200), B27 (1/100) (Life Technologies), 1 mg/ml laminin (Sigma), 10 ng/mL EGF and FGF-2 (Peprotech), 1Å~ MEM NEAA (Gibco), 0.1mM betamercaptoethanol, 0.012% BSA (Gibco), 0.2 g/L glucose (Sigma), 1000 U/ml penicillin-streptomycin (Sigma). Cells were dissociated using Accutase solution (Sigma).

- **ELISA displacement assay and Ki/$IC_{50}$ determination**

96- well ELISA high binding plates (Corning Costar, 9018) were incubated overnight at 4 °C with 100 µL/well of 1 µg/mL EphA2-Fc (R&D System, 639-A2) diluted in sterile phosphate buffered saline (PBS, 0.2 g/L KCl, 8.0 g/L NaCl, 0.2 g/L KH2PO4, 1.65 g/L Na2HPO4, pH 7.4). The day after, wells were washed with a washing buffer (PBS 0.05% tween20, pH 7.5) and blocked with a blocking buffer solution (PBS 1% BSA) for 1 h at 37 °C. Compounds were added to the wells at a proper concentration in 0.5% DMSO and incubated at 37 °C for 1 h. Biotinylated ephrin-A1-Fc (R&D Systems, BT602) was added at 37 °C for 4 h at its $K_D$ value in displacement assays or in a range from 1 to 2000 ng/mL in saturation studies. After 4h for displacement assays

or 5 h for saturation studies, wells were washed and incubated with 100 µL/well Streptavidin-HRP (Sigma S5512) for 20 min at room temperature. Then, wells were washed again with a washing buffer and incubated at room temperature with 0.1 mg/mL tetramethylbenzidine (Sigma T2885) reconstituted in stable peroxide buffer (0,05M citric acid +0,05 M dibasic sodium phosphate, pH 5). The reaction was quenched with 3 N HCl. Absorbance was read at 450 nm (ELISA plate reader). The $IC_{50}$ and Ki values were determined using one-site competition nonlinear regression analysis with Prism software (GraphPad Software Inc.).

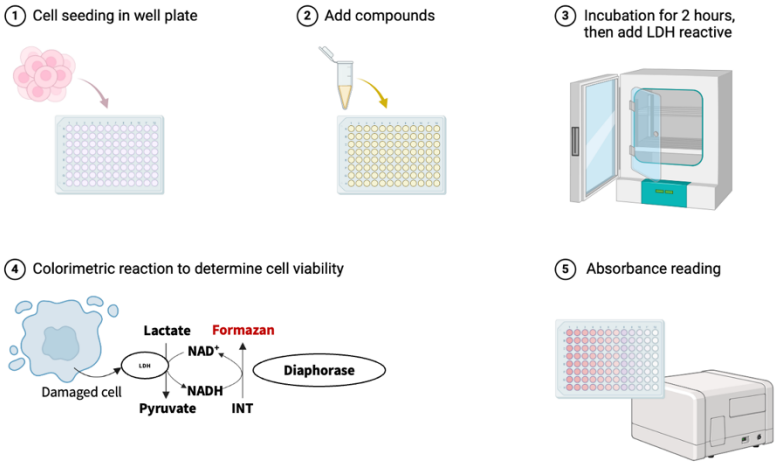



*Representative picture of ELISA displacement assay to calculate $IC_{50}$ and Ki*

- **<u>LDH assay</u>**

Non-specific toxicity induced by studied compounds on U251 cells was assessed measuring the release of Lactate Dehydrogenase (LDH) into the cell culture medium after two hours of incubation with compounds. The cytotoxicity was evaluated using CytTox 96® nonradioactive cytotoxicity assay (Promega, #1780) in order to identify the maximal safe concentration usable in cellular functional assays. Briefly, cells were seeded in 96-well plates (Falcon, #353072) at a concentration of 10(5) cells/ml and the day after were serum starved. Then, the cells were treated with compounds at 50, 25, 12.5 µM, DMSO 0.5% or lysis solution (9% v/v Triton-X 100). After 2 h of incubation, the culture medium was aspirated and centrifugated at 500 g for 4 min to obtain a cell free supernatant. LDH quantification was measured using 30 min coupled enzymatic assay which results in the conversion of iodonitrotetrazolium violet (INT) into red formazan. The amount of coloration observed is proportional to the number of lysed cells and quantified at 492 nm using an ELISA plate reader (Sunrise, TECAN, Switzerland).

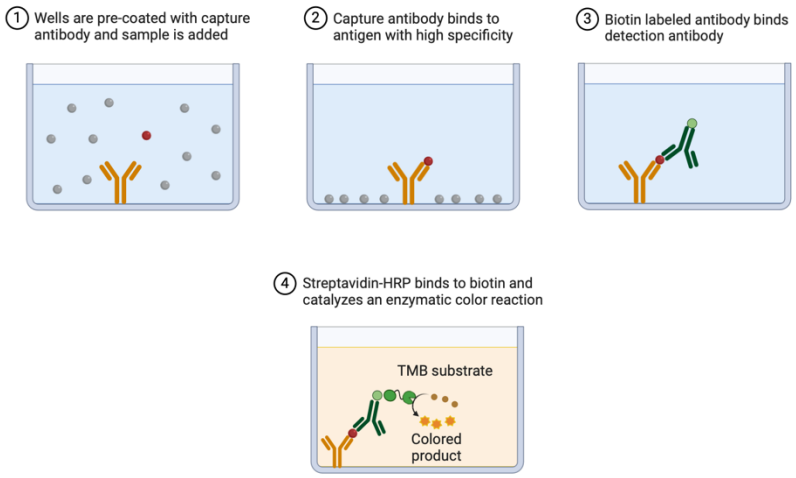
The results were expressed as the ratio between absorbance of the cells treated with compounds and treated with Lysis solution.




*Representative picture of LDH assay*

- **<u>Cell lysates and EphA2 phosphorylation</u>**

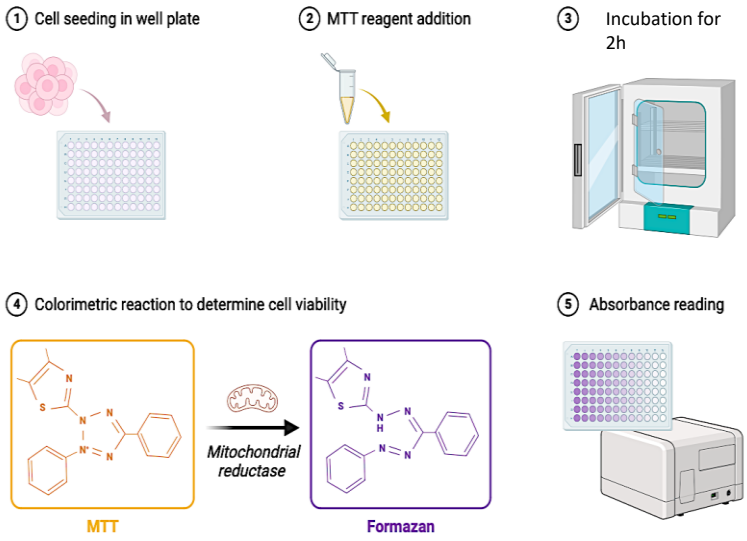
Cells were seeded in 12-well plates (Corning Costar, #3513) at the concentration of 7*10(4) cells/ml, 1 ml/well, in complete medium and incubated overnight. The day after, cells were starved overnight. Next, cells were treated with studied compounds, vehicle or standard drug, stimulated with ephrin-A1-Fc 0.1 µg/ml or not, rinsed with sterile PBS and solubilized in lysis buffer (1% NP-40, 20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM activated sodium orthovanadate, 10 µg/mL aprotinin, and 10 µg/mL leupeptin). The lysates were resuspended and rocked at 4 ◦C for 30 min and then centrifugated at 2000xg for 5 min at 4 ◦C. The protein content of supernatant was measured with BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA, #23250). EphA2 phosphorylation was measured in cell lysates using a DuoSet® IC Human Phospho-EphA2 ELISA Kit (R&D Systems, #DYC4056) following the manufacturer's protocol. Briefly, 96-well ELISA high binding plate (Corning Costar, #9018) was incubated overnight at room temperature with 100 µl/well of EphA2 capture antibody diluted in sterile PBS to the proper working concentration. After blocking, the wells were incubated for 2 h at room temperature with 100 µl/well of lysates, followed by 2 h incubation at room temperature with the detection antibody diluted in sterile PBS + 0.1% BSA. EphA2 phosphorylation was revealed utilizing a standard HRP format with a colorimetric reaction read at 450 nm by an ELISA plate reader (Sunrise, TECAN, Switzerland).




*Representative picture of ELISA assay*

- **<u>MTT assay</u>**

Antiproliferative and/or cytostatic capacity of the compounds were tested by using MTT assay on the different GBM cells line at different time point: 24,48 and 72h. Briefly cells were seeded, incubated for 24h at 37°C. After incubation compounds were added and at each time point MTT solution (1mg/ml, 100µL/wells) was added and incubated for 2h with the cells. The measure of samples absorbance was done by an ELISA plate reader (Sunrise, TECAN, Switzerland). The wavelength used to measure absorbance is 550 nm.




*Representative picture of MTT assay*

- ***In vitro* immunohistochemistry**

Internalization of EphA2 receptor:

Cells were fixed in 4% PFA, permeabilised in 0.5% Triton X-100 and blocked in 10% serum in PBS then incubated with primary antibody overnight at 4 °C in PBS + 10% serum. Secondary antibodies were diluted in 10% serum in PBS and incubated at RT for 1 h. Coverslip were mounted with VECTASHIELD® Antifade Mounting Medium with DAPI (VectorLab; H-1200-10) for 20 minutes. Imaging was carried out using the LEICA Stellaris 5. Images analysis was performed using Fiji ImageJ.

- **Flow cytometry analysis**

FACS analysis was performed through Click-iT™ EdU Alexa Fluor™ 488 Flow Cytometry Assay Kit (C10420; Thermofisher).

Cells were fixed in 4% PFA, permeabilised in 1% Triton X-100 and blocked in 5% BSA in PBS then incubated with Click-IT cocktail for 30' at room temperature. Then the samples were stained with a solution of Triton 0,5% + Dapi (1:10.000 in PBS). Analysis of EdU incorporation was performed using BD Fortessa X20. Data analysis was performed using FlowJo software.

- ***In vivo* pharmacokinetic**

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 intra peritoneal administration of compounds to 8 weeks old CD1 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 heart puncture (post-mortem) 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 29 software (Thermo Italia, Milan, Italy) was used for sample injection, peaks integration, and plasma level quantification.

# RESULTS

- **Characterization of new generation of Δ5-cholenic acid derivative**

Identification of the first Eph-ephrin antagonist derived from bile acids is UniPR1331, distinguished by a lack of biological activity toward classical targets of bile acids, including the G protein-coupled receptors TGR5 and the nuclear receptor FXR. Further studies revealed that. Apart from binding EphA2, UniPR1331 has also been shown to bind additional receptors, such as EphB2, and it has also been shown to directly bind VEGFR2[106].

Thanks to the collaboration with Prof. Alessio Lodola research group, a second generation of Δ5-cholenic acid derivatives was generated. As a result, an indole N-H group of UniPR1331 points towards an accessory pocket at the LBD of EphA2 from docking studies.

The docking simulations suggested that, while the 3β-hydroxy-Δ5-cholenic acid moiety of UniPR1331 targets a generally lipophilic and buried region of the LBD in both receptors, the L-tryptophan moiety targets an accessory and solvent-exposed pocket in EphA2.

These simulations suggested that substituent at the N-H indole group in the peptide would lead to improved interaction with EphA2 through an aromatic substituent of appropriate linkage that can fit an auxiliary pocket defined by Met55 and Asn71 residues.

Such chemical manipulation of the N-H group with appropriate substituents and a sulfonylbenzene group from an L-β-homo tryptophan analogue of UniPR1331 may present compounds against EphA2 isoform-targeting molecules to ensure enhanced selectivity.

The first synthesized compound was UniPR1447, the L-β-homotryptophan analogue of UniPR1331. Incorporation of L-β-homotryptophan instead of L-tryptophan has the advantage that chemical manipulation can take place without risking racemization because the indole N-substitution reaction conditions involve strong bases that could invert the stereochemistry at the Cα position during pH neutralization.

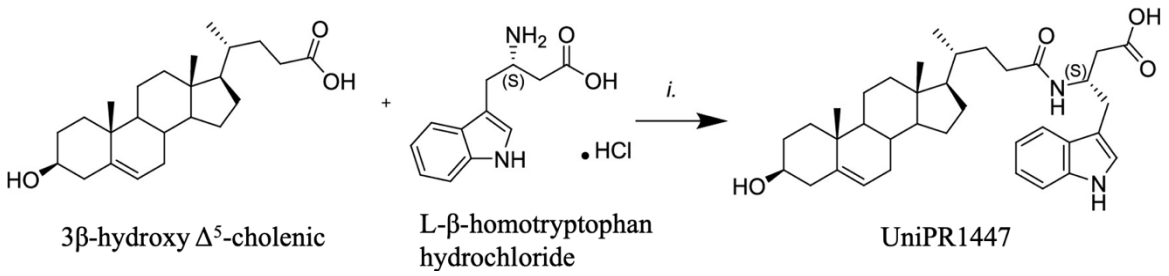
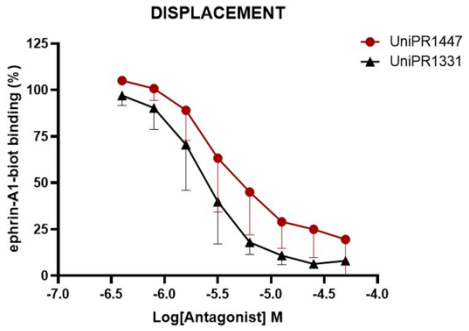



***Fig.6*** *Reagents and Conditions: (i) DIPEA 5 equiv, TBTU 1.2 equiv, Anhydrous. DMF, 0 °C, 30 min.*

The UniPR1447 binding behaviour against EphA2 was explored through an ELISA–binding assay. It was performed to explore the inhibitory potential of UniPR1447 on the binding of biotinylated ephrin-A1-Fc to the EphA2 receptor. The displacement curves were plotted by varying the ligand concentrations from 0,39 to 50 µM in the presence of biotinylated ephrinA1-Fc at its $K_D$ value. Looking at the curves, it was observed that UniPR1447 exhibited concentration-dependent inhibition against EphA2-ephrin-A1 binding. Indeed, $IC_{50}$ values of UniPR1447 against the reference antagonist UniPR1331 have been reported to be about 6,6µM and 2,6µM respectively[107]. Hence, based on the results of this assay, UniPR1447 showed a very close value of $IC_{50}$ compared to its references compound UniPR1331.

**A**




**B**

| $IC_{50}$ | Value (µM) |
|---|---|
| UniPR1447 | **6,6** [4.6 – 10] |
| UniPR1331 | **2.6** [2.0 – 3.3] |

***Fig.7*** *Binding curves for UniPR1447 and UniPR1331 (A) in the displacement assay of biotinylated ephrin-A1-Fc from EphA2-Fc. Compounds at the indicated concentrations and 0.5% DMSO (used as control) were added in the wells. Then they were incubated for 1 h at 37 °C. To the wells were added biotinylated ephrin-A1-Fc and further incubated for an additional 4 h at 37 °C. Table showing the $IC_{50}$ value with standard deviation for the compounds UniPR1447 and UniPR1331(B).*

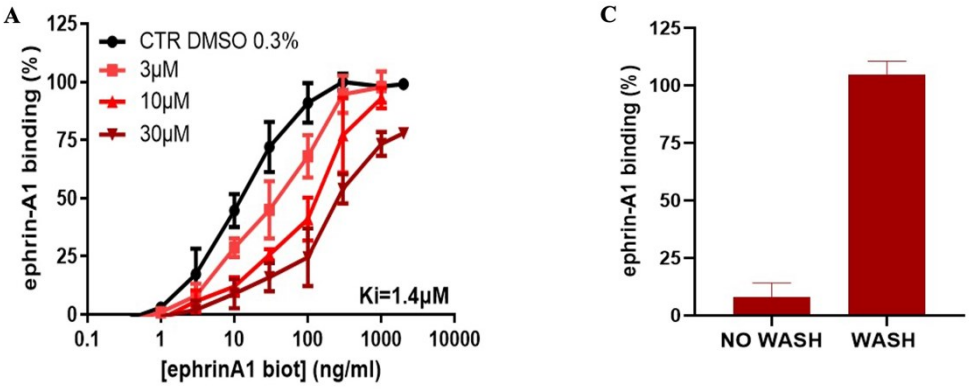
The next step was to carry out a saturation binding assay. It was performed to identify interference of UniPR1447 on the ephrin-A1/EphA2 interaction.

This was carried out by using increasing concentrations of the natural ligand ephrin-A1 in the presence of different concentrations of the compounds or the vehicle alone. The saturation binding assay is an important method essential to understand the type of interaction between the and the receptor binding site.

By conducting this test, I was able to determine the Ki values, helping us to further understand the affinity and characteristics of the compounds' binding. The Ki value of UniPR1447 was found to be 1.4µM. Furthermore, when examining the UniPR1447 curve, it caused a concentration-related shift to the right in saturation binding curves of biotinylated ephrin-A1. This shift to the right could be interpretate as a surmountable inhibition, whereby the molecule competes with the natural ligand for the same binding site on the receptor. Essentially, higher concentrations of natural ligand overcome the binding of the molecule.

UniPR1447 turned out being reversible binders for EphA2 receptor, as demonstrated by the restored biotinylated ephrin-A1 binding in wells after inhibitor wash out.

Taken together this data suggest UniPR1447 is a reversible and competitive ligand.




B

| Parameter | Value | Units |
|---|---|---|
| $K_D$ (ephrin-A1) | **15** | ng/mL |
| Ki (UniPR1447) | **1.4** [1.034 - 1.8] | µM |

***Fig.8*** *Saturation curves of biotinylated ephrin-A1-Fc to immobilized EphA2 (A) in the presence of increasing concentrations of UniPR1447. Table showing the $K_D$ data, obtained using the ephrin-A1 ligand, and the Ki value with standard deviation for the compound UniPR1447(B). Reversibility assay: EphA2–ephrin-A1 binding in the presence of 50 µM UniPR1447 with or without washing three times with PBS before the addition of biotinylated ephrin-A1-Fc (C)*

- **Evaluation of antagonist behaviour of UniPR1447**

To determine whether the compound tested was acting as antagonist or agonist, a phosphorylation assay using U251 glioblastoma cells was performed, which endogenously overexpress the EphA2 receptor.

The test compound was thus pre-incubated with U251 cells for 20 minutes, after which the cells were stimulated with ephrin-A1-Fc at a concentration of 0.1 µg/ml for 10 minutes. The cells were then lysed, and the extents of phosphorylation of the EphA2 receptor quantified by an ELISA assay.

Conversely, to measure agonistic activity, the cells were incubated with the compound alone for 20 minutes before lysis. The phosphorylation detected was compared to a maximal phosphorylation reference value (100%) that was arbitrarily set as the phosphorylation in cells treated with the vehicle, 0.3% DMSO, for 20 minutes and then stimulated with 0.1 µg/ml ephrin-A1-Fc. On the other hand, the UNSTIM condition refers to cells that were treated only with the vehicle, 0.3% DMSO, for 20 minutes and were not further stimulated.

UniPR1447 significantly inhibited phosphorylation of the EphA2 receptor at 30 µM and 10 µM. This may indicate that the compound exerts antagonistic activity against the EphA2 receptor in U251 GBM cells, thus supporting their viability as therapeutic agents in the treatment strategies of glioblastoma targeting EphA2.

Moreover, compound did not show agonistic properties, in fact, no signal was detected when U251cells were treated only with compounds without ligand stimulation, confirming that they act as antagonist.

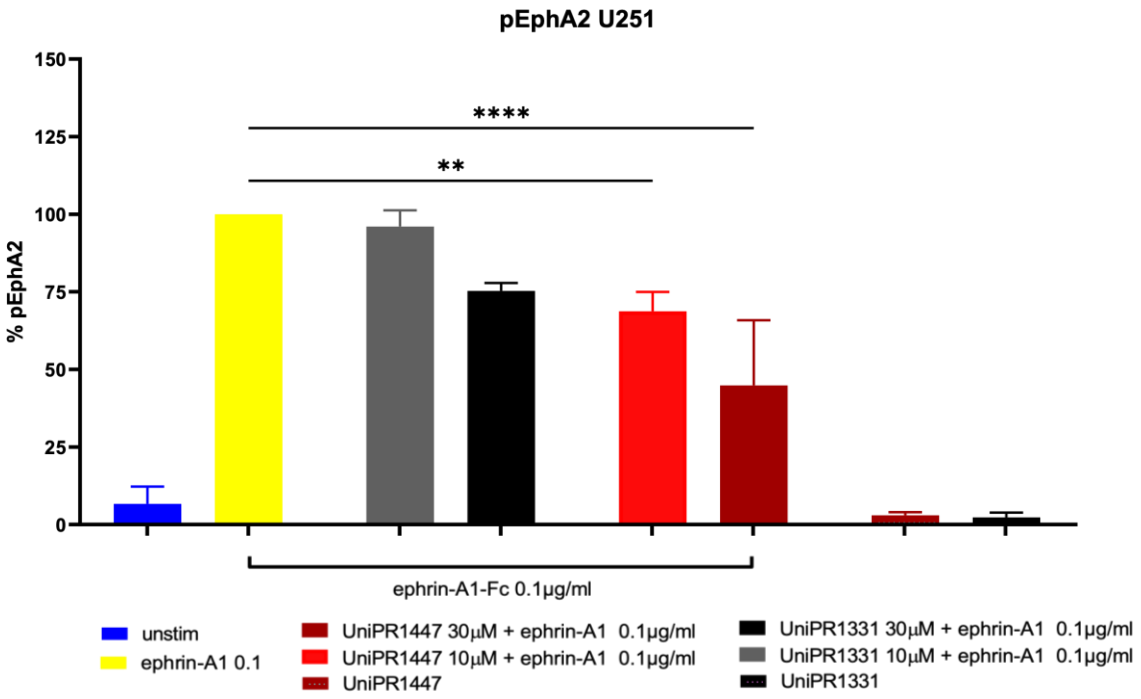



***Fig.9*** *Phosphorylation of EphA2 in U251 cells. Cells were pretreated for 20 min with 0.3% DMSO or with the compounds at the indicated concentrations and then stimulated for 10 min with ephrin-A1-Fc 0.1µg/ml. Phospho-EphA2 levels are relative to ephrin-A1-Fc + DMSO. One-way ANOVA followed by Tukey post-test was performed to compare ephrin-A1-Fc + DMSO to all the other columns and control to all other columns. **p < 0.01, **** p<0.0001. Data are the means of at least 3 independent experiments ± SD.*

Because an inhibition of the EphA2 activation could be ascribed to a non-specific cytotoxic effect, LDH assay was performed on U251 cells, by testing compound at concentrations capable to block the phosphorylation of the receptor.

This cell-based assay thus measures the enzyme LDH in the cell culture medium as an indication of potential nonspecific toxicity. LDH is normally localized within the cytoplasm; hence, its presence in the culture medium could mean membrane damage, which is due to its exposure to cytotoxic agents.

As indicated by the graph below (fig.10), none of these tested compounds induced a significant amount of LDH release at 30 μM following 2-hour incubation with U251 cells cultured in serum-free culture medium. For reference, the LDH release from cells treated with the vehicle and a cell lysis buffer was defined to represent 0% and 100% cytotoxicity, respectively.

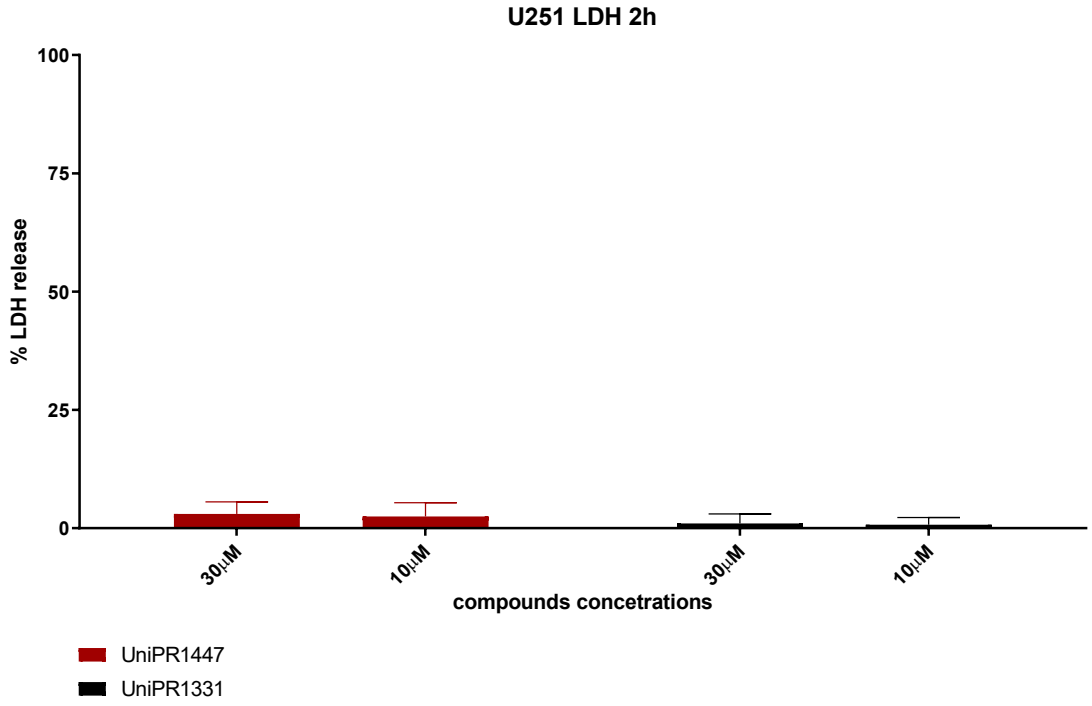



***Fig.10*** *Relative levels of LDH after 2h of incubation with compounds.*

- **<u>The modification of indole group led to the synthesis of new compound: UniPR1449</u>**

As reported above, thanks to the docking simulations it was clear that substituent at the N-H indole group in the peptide would lead to improved interaction with EphA2 through an aromatic substituent of appropriate linkage that can fit an auxiliary pocket defined by Met55 and Asn71 residues. So, based on this observation it was synthetized, by professor Lodola research group, another compound: UniPR1449

Synthesis of UniPR1449 had to be accomplished in several steps from commercially available L-β-homotryptophan hydrochloride. First, the carboxyl group had to be esterified to its methyl ester. This intermediate was then converted to the corresponding tert-butyl carbamate protected form. The subsequent sulfonylation of Boc-protected L-β-homotryptophan was done under conditions of phase transfer and introduced a sulfonyl group at the indole nitrogen. After that, the obtained N-indole-sulfonylated compound was deprotected by the removal of the Boc-group. The obtained intermediate was condensed without additional purification with 3β-hydroxy Δ5-cholenic acid using the coupling reagent 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate (TBTU). Hydrolysis of the methyl ester ended with the target compound UniPR1449[107].

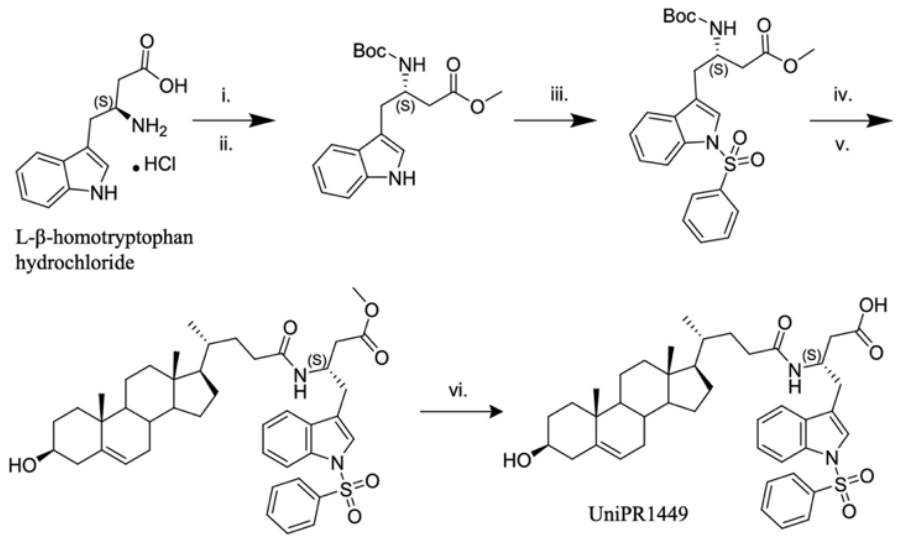
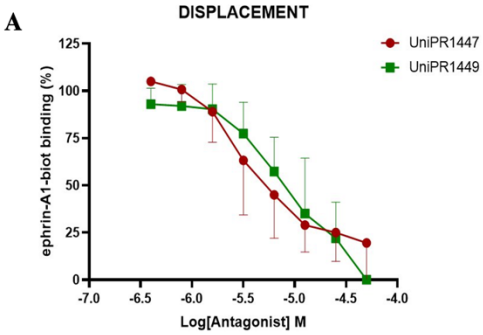



***Fig.11*** *Reagents and Conditions: (i) MeOH, SOCl2; (ii) Boc2O, NaHCO3, THF/H2O; (iii) PheSO2Cl, NaOH, N(Bu)4Cl, DCM/H2O; (iv) TFA/DCM, 0 °C; (v) 3β-hydroxy-Δ5cholenic acid, TBTU, DIPEA, DMF; (vi) LiHMDS, THF/H2O.*

As done for UniPR1447, the binding behaviour against EphA2 was explored with an ELISA–binding assay for UniPR1449. It has been performed to explore the inhibitory potential of UniPR1449 on the binding of biotinylated ephrin-A1-Fc to the EphA2 receptor. The compound was used with a range of concentrations from 0,39 up to 50µM in the presence of biotinylated ephrinA1-Fc at its $K_D$ value. As observed from the curves, UniPR1449 showed concentration-dependent inhibition against EphA2-ephrinA1 binding. $IC_{50}$ values of UniPR1449 were previously reported to be about 8,3µM[107]. Therefore, according to this result, UniPR1449 showed a very close value of $IC_{50}$ to its reference compound UniPR1447.




B

| $IC_{50}$ | Value (µM) |
|---|---|
| UniPR1449 | **8,3** [5.6 – 12] |
| UniPR1447 | **6,6** [4.6 – 10] |

***Fig.12*** *Binding curves for UniPR1447 and UniPR1449 in the displacement assay of biotinylated ephrin-A1-Fc from EphA2-Fc. Compounds at the indicated concentrations and 0.5% DMSO (used as control) were added in the wells. Then they were incubated for 1 h at 37 °C. To the wells were added biotinylated ephrin-A1-Fc and further incubated for an additional 4 h at 37 °C. Table showing the $IC_{50}$ value with standard deviation for the compounds UniPR1449 and UniPR1447 (B).*

A saturation binding assay was then conducted after the synthesis of UniPR1449 to determine whether UniPR1449 interferes with the ephrin-A1/EphA2 interaction. This will be done by varying the concentrations of the natural ligand ephrin-A1 in the presence of UniPR1449 to determine its effect on the binding affinity of ephrin-A1 to EphA2. The Ki for UniPR1449 was calculated from these experiments with a Ki value of 2.2 µM.

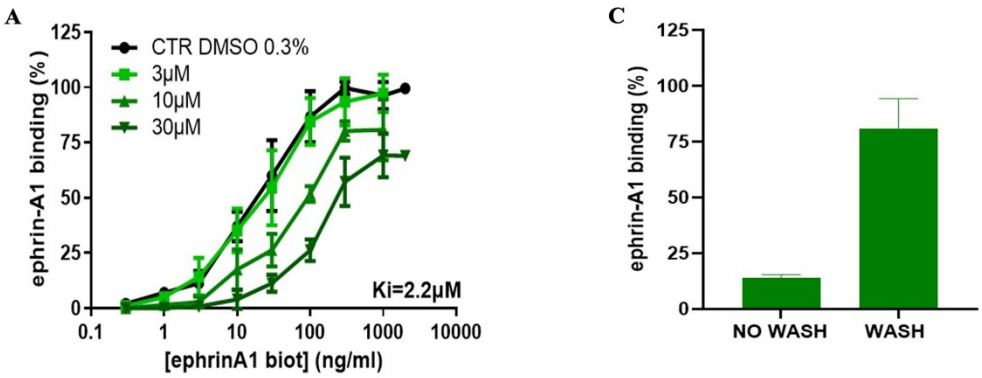
Moreover, saturation binding curves of biotinylated ephrin-A1 were rightwardly shifted by UniPR1449 in a concentration-dependent manner. This clearly indicates surmountable binding, whereby the molecule competes with the natural ligand for the same binding site on the receptor.

As indicated from the graph, at a concentration of 30 µM, the saturation curve shifts to the right while at the same time decreasing.

In order to explore if the interaction of UniPR1449 with the LBD of EphA2 was reversible or irreversible a reversibility assay was conducted.

It was shown that UniPR1449 reversibly interacts with the EphA2 receptor. This was attested to by the recoverage of the binding of biotinylated ephrin-A1 to EphA2 in wells after the inhibitor had been washed out, which confirms that UniPR1449 does not irreversibly bind or permanently modify the binding site.

Taken together this data suggest UniPR1449 is a reversible and competitive ligand.




B

| Parameter | Value | Units |
|---|---|---|
| $K_D$ (ephrin-A1) | **15** | ng/mL |
| Ki (UniPR1449) | **2.2** [1.7 – 3.0] | µM |

***Fig.13*** *Saturation curves of biotinylated ephrin-A1-Fc to immobilized EphA2 (A) in the presence of increasing concentrations of UniPR1449. Table showing the $K_D$ data, obtained using the ephrin-A1 ligand, and the Ki value with standard deviation for the compound UniPR1449 (B). Reversibility assay: EphA2–ephrin-A1 binding in the presence of 50 µM UniPR1449 with or without washing three times with PBS before the addition of biotinylated ephrin-A1-Fc (C).*

- **Evaluation of antagonist behaviour of UniPR1449**

Additionally, the potential antagonistic or agonistic activity of UniPR1449 on the EphA2 receptor was assessed using the same *in vitro* assay performed for UniPR1447.

UniPR1449 significantly inhibited phosphorylation of the EphA2 receptor at 30 µM. Moreover, compound did not show agonistic properties, in fact, no signal was detected when U251 cells were treated only with compounds without ligand stimulation, confirming that they act as antagonist.

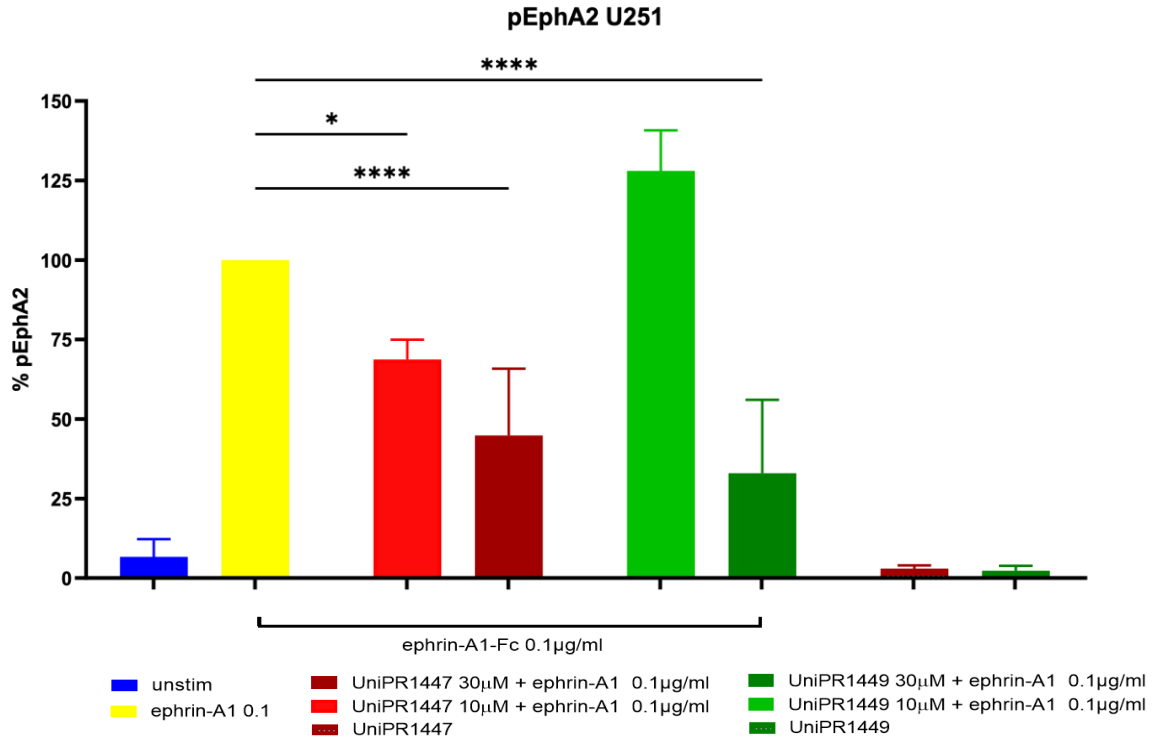



***Fig.14*** *Phosphorylation of EphA2 in U251 cells. Cells were pretreated for 20 min with 0.3% DMSO or with the compounds at the indicated concentrations and then stimulated for 10 min with ephrin-A1-Fc 0.1µg/ml. Phospho-EphA2 levels are relative to ephrin-A1-Fc + DMSO. One-way ANOVA followed by Tukey post-test was performed to compare ephrin-A1-Fc + DMSO to all the other columns and control to all other columns. *p < 0.05, **** p<0.0001 Data are the means of at least 3 independent experiments±SD.*

To exclude the possibility that the inhibition of the phosphorylation process observed in these experiments was due to some non-specific effects, an additional assay of lactate dehydrogenase was done. This assay was conducted under the same conditions as the previous experiments. The compounds were tested at 30 and 10µM. The data obtained from this assay revealed that neither UniPR1447 nor UniPR1449 exerted a significant release of LDH, hence excluding any non-specific cytotoxicity as the cause for the inhibition of EphA2 phosphorylation (fig.15).

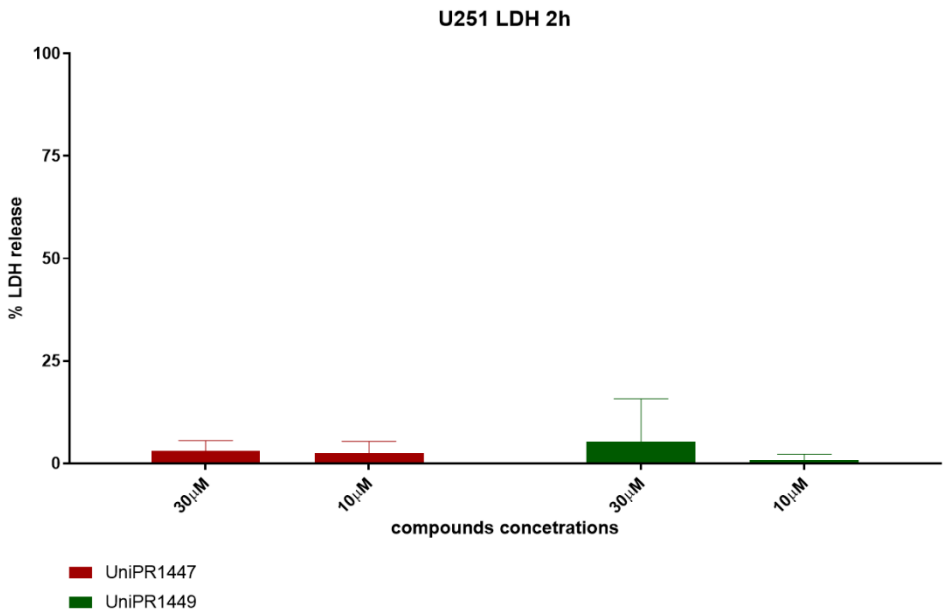



***Fig. 15*** *Relative levels of LDH after 2h of incubation with compounds.*

- **Determination of UniPR1447 and UniPR1449 Kinase activity**

Moreover, to evaluate whether UniPR1447 and UniPR1449 directly inhibit the kinase domain of EphA2, an enzyme-based assay was conducted. In this assay, recombinant EphA2-kinase was incubated with a specific substrate, Ulight-TK peptide (50 nM). Phosphorylation of this substrate by the kinase leads to recognition by a Europium-labeled anti-phospho antibody, resulting in light emission detected through the LANCE (Lanthanide Chelate Excitation) method. This assay was carried out by Eurofins Discovery (fig.16).

The results indicated that neither UniPR1447 nor UniPR1449 altered the kinase activity of EphA2. This suggests that these compounds do not directly inhibit the kinase domain of EphA2 but likely interfere with the receptor's interaction with its natural ligand, ephrin-A1. By confirming that these compounds do not affect kinase activity, it can be inferred that their antagonistic effects are due to their binding to the ligand-binding domain (LBD) rather than the kinase domain.

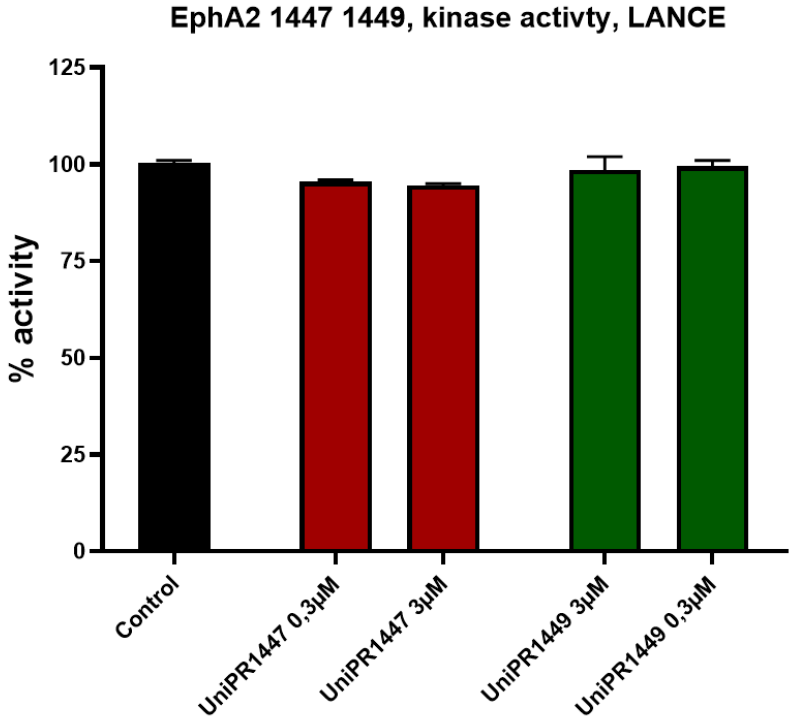
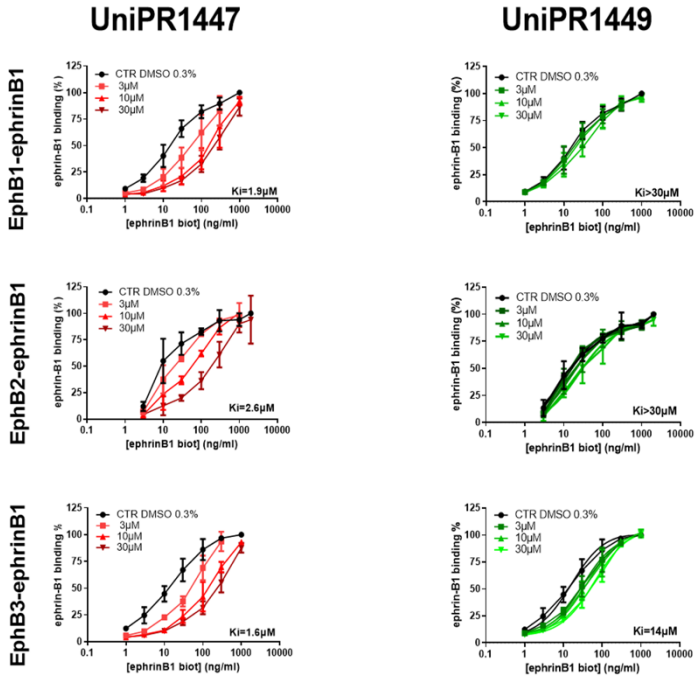



***Fig. 16*** *Recombinant human EphA2 enzyme activity was evaluated with LANCE® method using ATP and Ulight-TK peptide as substrate. Human EphA2 kinase was previously incubated with 0,3 and 3 µM UniPR1447 or UniPR1449, 1% DMSO (control) for 30 minutes.*

- **<u>Study of UniPR1447 and UniPR1449 selectivity on EphBs family</u>**

As previously mentioned, the general idea behind synthesizing these compounds was to increase their selectivity for the EphA2 receptor relative to UniPR1331. To this end, I decided to explore the selectivity on Eph-B family receptors. The present study employed saturation binding experiments that characterized the affinity of these compounds with different Eph receptor-ligand pairs. As reported in the fig.17 UniPR1447 did not discriminate between the members of EphB receptors family and it bound promiscuously EphA and EphB subclasses, thus, acting as pan-inhibitor. Conversely, UniPR1449 did not show any activity on EphB receptors family.

**A**




**B**

| Ki (µM) | UniPR1447 | UniPR1449 |
|---|---|---|
| EphB1-ephrinB1 | **1.9** [1.3 – 2.6] | **>30** [25 - 98] |
| EphB2-ephrinB1 | **2.6** [1.8 – 3.7] | **>30** [18 – 60] |
| EphB3-ephrinB1 | **1.6** [1.2 – 2.0] | **14** [9.8 – 23] |

***Fig.17*** *Saturation curves of biotinylated ephrin-B1-Fc to immobilized EphB1/2/3 in the presence of increasing concentrations of compounds (A). Table showing the Ki value with standard deviation for the compound UniPR1447 and UniPR1449 (B).*

These results pointed out, among the two compounds, different binding affinities and specificities toward EphB2. More specifically, the remarkable activity of UniPR1447 against EphB receptors, together with the lack of activity of UniPR1449, outlined the role of structural modifications at the level of the L-β-homotryptophan indole in terms of receptor selectivity. This is of relevance since it has already been reported that variations in the indole moiety might be exploited in the design of ligands with improved selectivity for EphA2.

- **EphA2 degradation**

After assessing the two compounds for inhibition of phosphorylation without non-specific toxicity, I performed another assay to explore better the antagonistic activity against EphA2 and to verify their interaction with the LBD of the receptor.

As reported in literature the cell membrane-expressed EphA2 receptor is internalized and then degrade upon being stimulated by its ligand, ephrin-A1[108]. In the presence of the compounds, I did this experiment to see if the receptor would still internalize and degrade, thus confirming that the compounds act as antagonists.

I first performed this assay using a commercial drug, Dasatinib (fig.18). I decide to use it since it is a tyrosine kinase inhibitor that does not interact with the LBD of the receptor and therefore does not prevent receptor internalization. This was used as a control to show that internalization of the receptor requires interaction with the ligand-binding domain of EphA2. Similar data have been reported by where dasatinib only partially reduced internalization on different pancreatic cancer cell lines[86].

The graph (fig.19) clearly shows: the EphA2 receptor without ligand is not subjected to internalization (Unstim). In the presence of ligand, the receptor is subjected to internalization. In absence of ephrin-A1, dasatinib did not internalize the receptor; on the other hand, in presence of ephrin-A1, dasatinib can not block EphA2 internalization. Based on these results it is true to affirm that the portion of LBD is implicated in internalization.

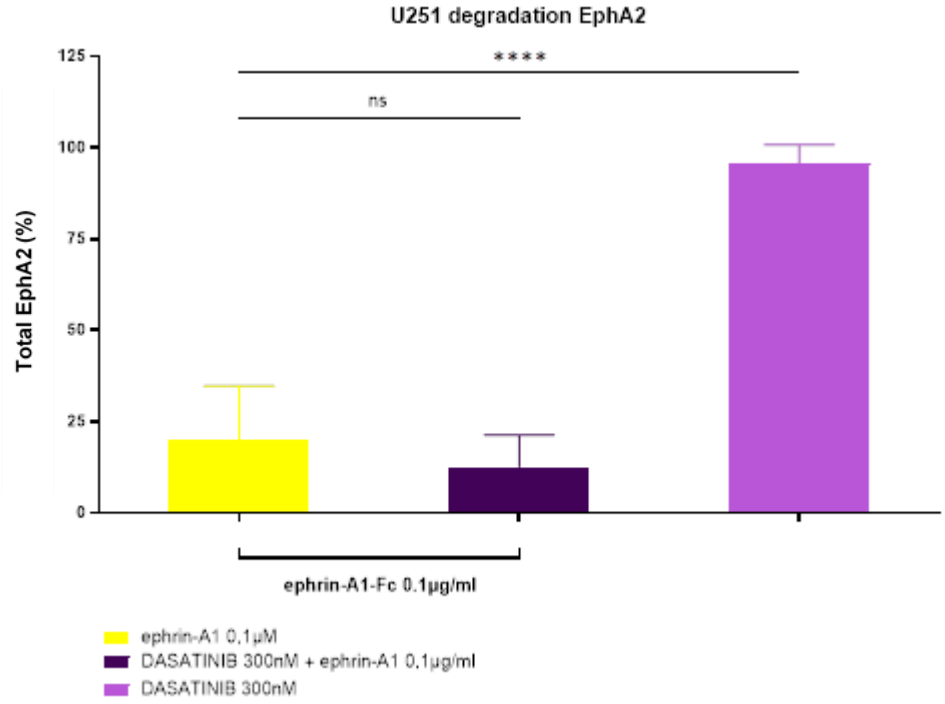



***Fig.18*** *Degradation of EphA2 in U251 cells. Cells were pretreated for 20 min with 0.3% DMSO or with the compound at the indicated concentrations and then stimulated for 4 hours with ephrin-A1-Fc 0.1µg/ml. EphA2 levels are relative to unstim condition (DMSO 0,3%; column not shown). One-way ANOVA followed by Tukey post-test was performed to compare ephrin-A1-Fc + DMSO to all the other columns. *p < 0.05, **p < 0.01. Data are the means of at least 3 independent experiments ± SD.*

Having obtained these preliminary data, I repeated the experiment with UniPR1447 and UniPR1449. This experiment consisted of treating the cells with both compounds at two different concentrations: 30 µM and 10 µM, in the presence of ephrin-A1.

As illustrated in the graph, these compounds strongly inhibit receptor internalization in the presence of ephrin-A1.

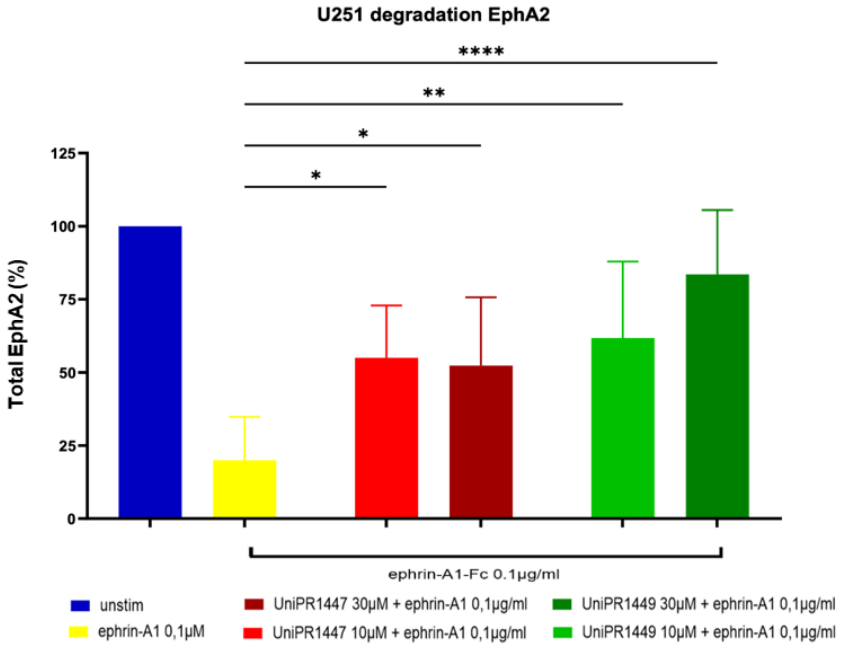



***Fig.19*** *Degradation of EphA2 in U251 cells. Cells were pretreated for 20 min with 0.3% DMSO or with the compounds at the indicated concentrations and then stimulated for 4 hours with ephrin-A1-Fc 0.1µg/ml. EphA2 levels are relative to unstim condition (DMSO 0,3%). One-way ANOVA followed by Tukey post-test was performed to compare ephrin-A1-Fc + DMSO to all the other columns. *p < .05, **p < 0.01, *** p<0.001, **** p<0.0001. Data are the means of at least 3 independent experiments±SD.*

- **EphA2 internalization**

To further demonstrate their antagonistic activities, I carried out an assay that shows the abilities of the two molecules to block the internalization of the EphA2 receptor. This was done by immunofluorescence techniques. A summary of the procedure is:

Cells seeded in appropriate coverslip culture conditions, upon adherence were treated with 0.1 µg/ml of ephrin-A1 (or vehicle: PBS) for two hours to induce receptor internalization. The treated cells were fixed using a paraformaldehyde solution and permeabilized using an appropriate agent like Triton X-100, to allow the antibody access to intracellular structures overnight incubation with a primary antibody, specific to EphA2, followed by 1 hour incubation with the fluorescently tagged secondary antibody was performed. The images of the coverslips are taken up with confocal microscope for the localization of the receptor, EphA2.

My first step was to perform the assay in the absence of compounds to verify that the EphA2 receptor is internalized into the cell in the presence of its natural ligand, ephrin-A1. This control experiment was important for enabling a comparison to be made of the effects of the compounds with respect to the baseline behaviour of the receptor toward its ligand.

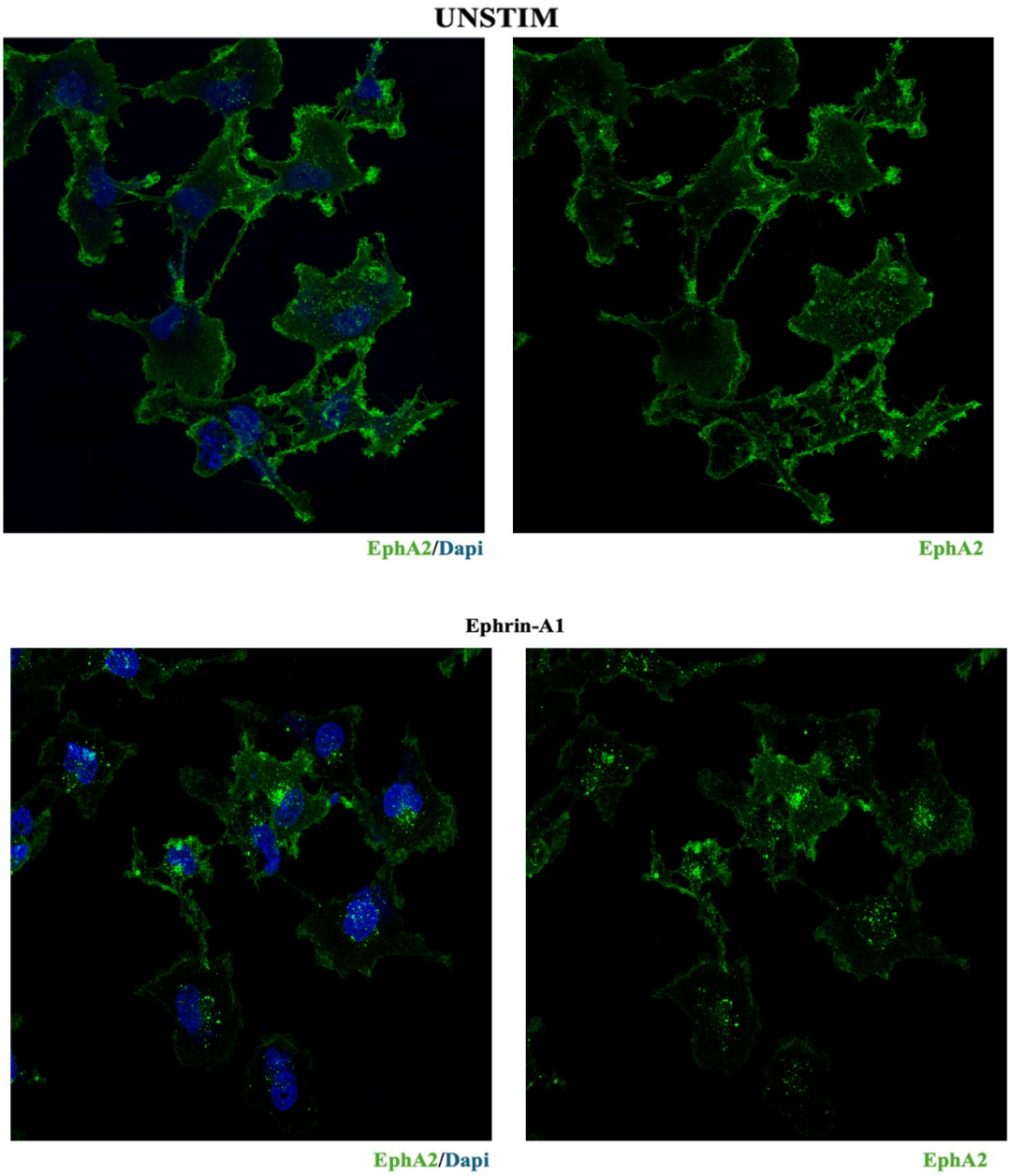



***Fig.20*** *Internalization of EphA2 in U251 cells. Cells were pretreated for 20 min with 0.3% DMSO then stimulated for 2 hours with ephrin-A1-Fc 0.1μg/ml or for the UNSTIM condition with the veichle (PBS). Images of the coverslips are taken up for confocal microscope for the localization of the receptor, EphA2.*

As indicated in the figures we can observe that:

• **Vehicle Control** (UNSTIM): Fig. 20, the fluorescence tag is largely confined to the cell membrane, thereby demonstrating that EphA2 stays on the cell surface in the absence of ephrin-A1.

• **Ligand Stimulated Condition**: Upon incubation with ephrin-A1, a striking shift of the fluorescence signal from membrane to intracellular compartments is observed, thus demonstrating internalization of the EphA2 receptor; Fig. 20.

This differential localization, as visualized using immunofluorescence, underscores the dynamism of the receptor in response to ligand binding and provides a basis for comparison for the subsequent experiments using UniPR1447 and UniPR1449 (fig.21 and fig.22).

After verification that the ligand ephrin-A1 induces internalization of the EphA2 receptor, I performed the experiment in the same conditions described above but in the presence of compounds UniPR1447 and UniPR1449. Both compounds at 30 and 10 µM were able to inhibit the receptor internalization in a dose-dependent manner, as shown in images and as reported in the graph (Fig.23).

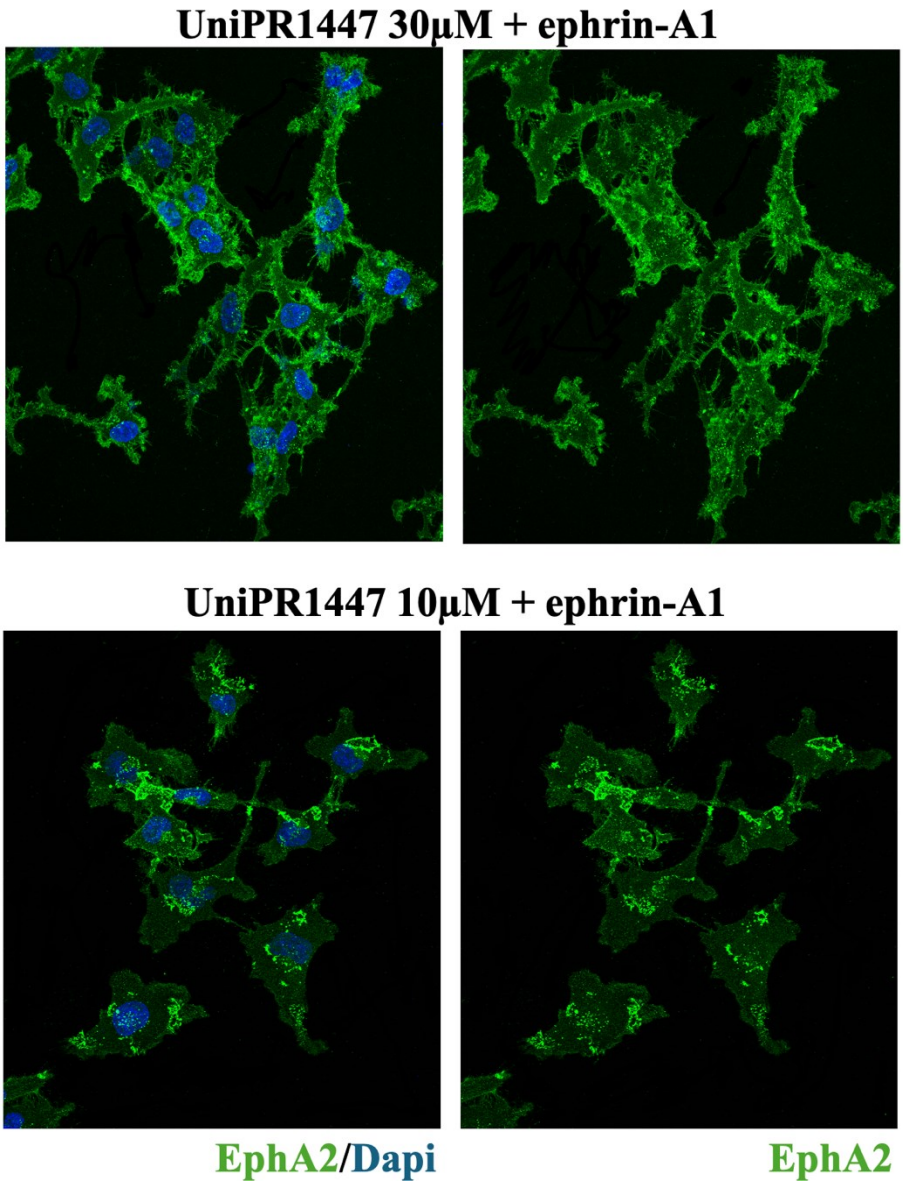



***Fig.21*** *Internalization of EphA2 in U251 cells. Cells were pretreated for 20 min with 0.3% DMSO or with UniPR1447 at the indicated concentrations and then stimulated for 2 hours with ephrin-A1-Fc 0.1µg/ml. Images of the coverslips are taken up for confocal microscope for the localization of the receptor, EphA2.*

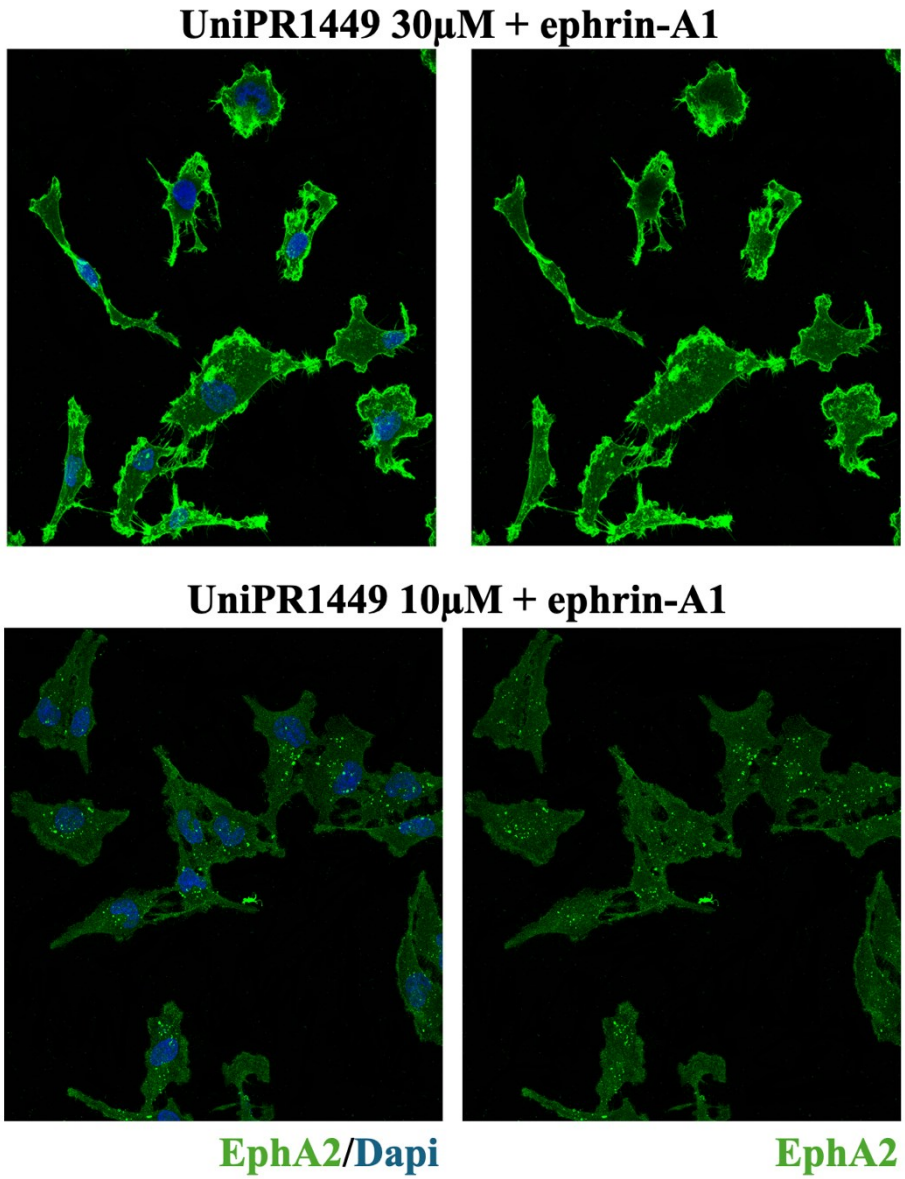



***Fig.22*** *Internalization of EphA2 in U251 cells. Cells were pretreated for 20 min with 0.3% DMSO or with UniPR1449 at the indicated concentrations and then stimulated for 2 hours with ephrin-A1-Fc 0.1µg/ml. Images of the coverslips are taken up for confocal microscope for the localization of the receptor, EphA2.*

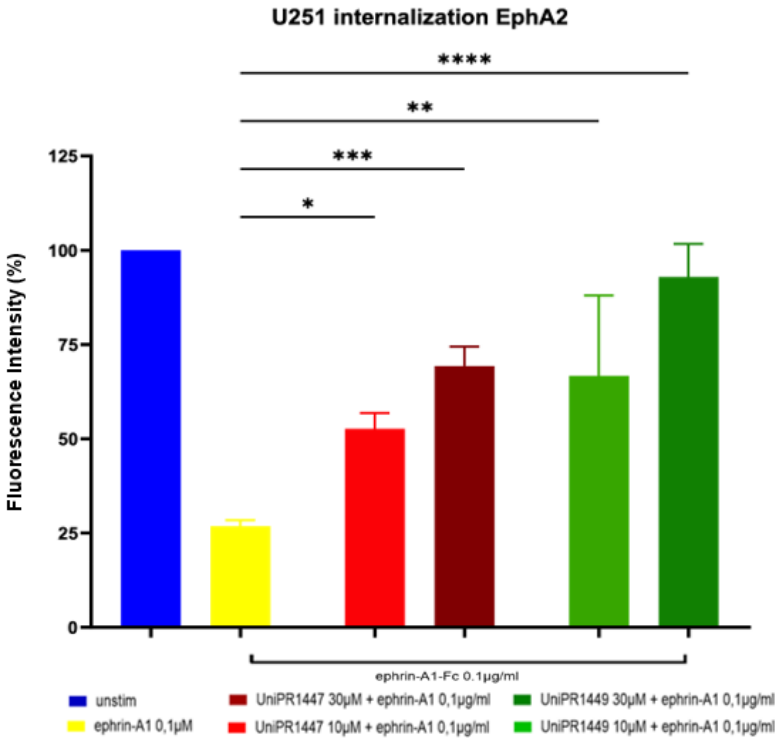



***Fig.23*** *Fluorescence was quantified using ImageJ software as corrected total cell fluorescence:* ***integrated density − (area of selected cell × mean fluorescence of background)****. Data are the mean ± S. D. of measurements on 25–35 cells for each sample from three independent experiments. One-way ANOVA followed by Tukey post-test was performed to compare ephrin-A1-Fc + DMSO to all the other columns. *p < 0.05, **p < 0.01, *** p<0.001, **** p<0. 0001.*

The compounds were also tested for agonistic activity by incubating them with the cells in the absence of the physiological ligand. Neither UniPR1447 nor UniPR1449 were capable of inducing receptor internalization (data not shown). This observation provides further confirmation of their antagonistic activity.

These results strongly point toward a dose-dependent antagonist action of both UniPR1447 and UniPR1449 upon the EphA2 receptor. This further validates the compounds' antagonistic properties and supports their use in disrupting EphA2-ephrin-A1 interactions in glioblastoma cell lines.

- **Proliferation studies: MTT assay**

The GBM U251 antiproliferative test was conducted using the MTT assay for each separated compound at different concentration from 30 to 3µM and different incubation periods at 24, 48, and 72 hours (fig.24).

The MTT assay is a colorimetric assay for assessing cell metabolic activity as an indicator of cell viability, proliferation, or cytotoxicity. The principle of this assay relies on the reduction of MTT by mitochondrial dehydrogenases to formazan, which is a purple dye quantified by absorbance measuring at 570 nm. The intensity of the colour produced directly correlates to the number of metabolically active cells.

For the incubation period as specified, the U251 cells in the experiment were treated with the compounds. Later, the MTT reagent put in each well was incubated further to form formazan crystals. These were then dissolved, and the absorbance was read against on a microplate reader.

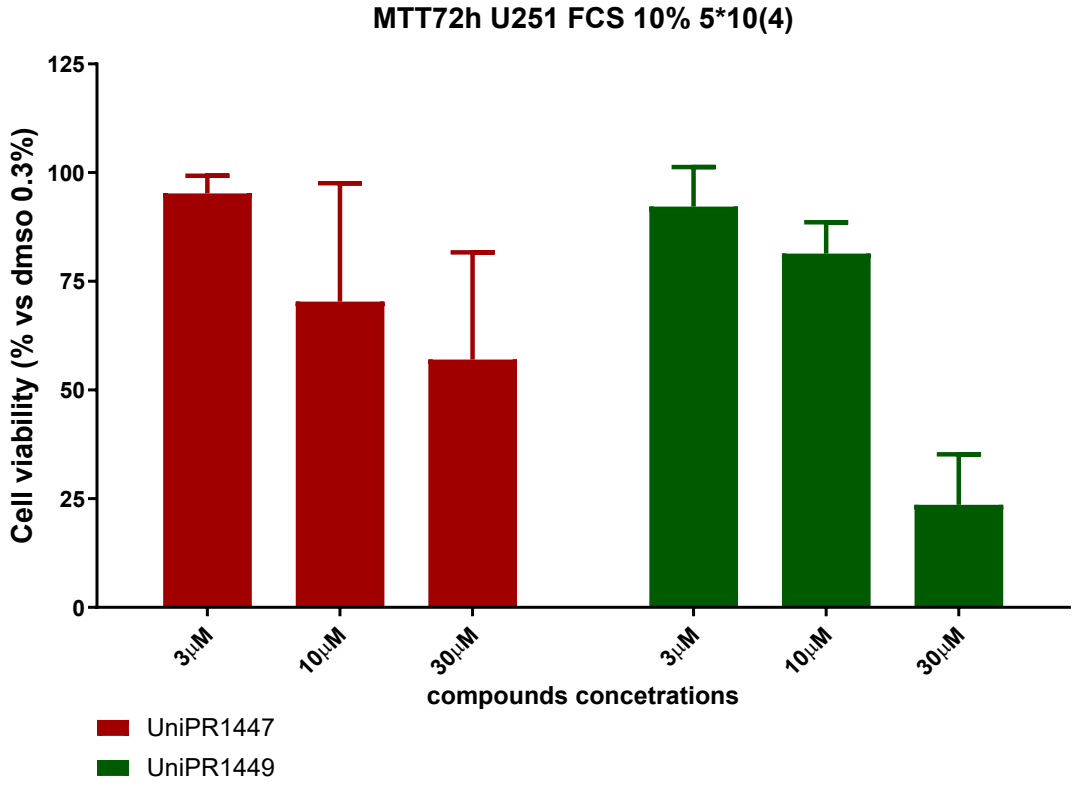



***Fig.24*** *Proliferation of U251 in presence of different concentrations of compounds. The assay was conducted at 24,48 (data not shown) and 72h with a range concentration from 3 to 30 µM.*

As illustrated in the fig.24, all compounds exhibit a dose-dependent ability to reduce proliferation. After 72 hours of incubation, both compounds demonstrated a significant reduction in the proliferation of the U251 cells when used at 30µM.

From the data obtained with the MTT assay, it was possible to construct growth curves, which are relevant to the elucidation of whether the antiproliferative activity observed is a cytostatic or cytotoxic effect (fig.25).

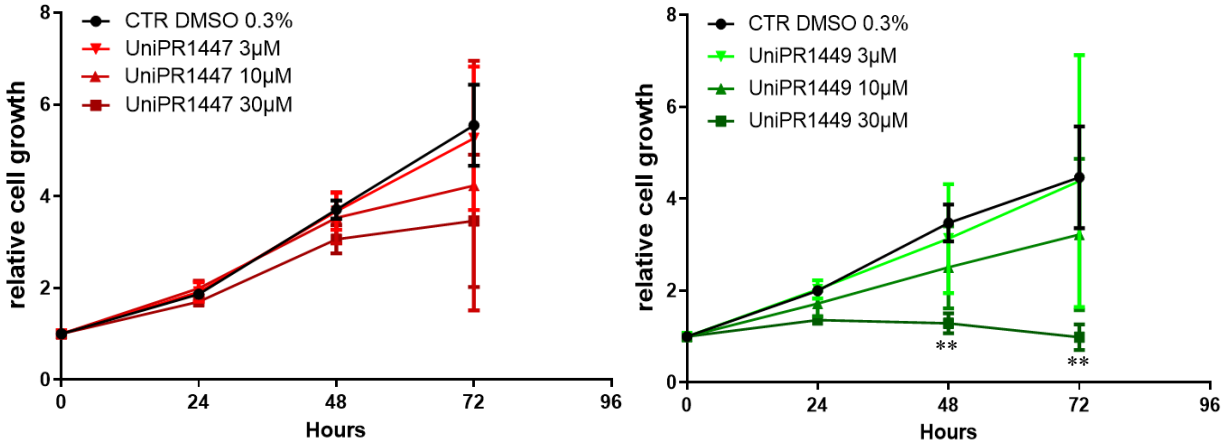



***Fig.25*** *Growth curves of U251 with and without the compounds. One-way ANOVA followed by Dunnet post-test was performed to compare control curve to all the other curves. *p <0.05, **p < 0.01, *** p<0.001, **** p<0.0001. Data are the means of at least 3 independent experiments ± SD.*

The growth curves modulate these two kinds of cellular responses. By cytostatic effect, it means that the growth and proliferation of cells have been inhibited; however, the induction of cell death is not observed, while this would be manifested by a plateau in the growth curve. On the other hand, a cytotoxic effect would be represented by a decline in the growth curve, indicating a reduction in cell number.

The two different modes of representation show that compound UniPR1449 is the most effective at reducing cell proliferation. In more detail, from the growth curves, at a concentration of 30 µM, one can notice that UniPR1449 cause a cytotoxic effect since the number of cells is reduced. At a lower concentration of 10 µM, UniPR1449 causes a cytostatic effect, manifested by the fact that it causes less cell growth, but not in number.

These observations underline the potency and dual-mode action of UniPR1449: it kills at higher concentrations and prevents proliferation at lower concentrations.

- **Study of Physico-chemical properties of UniPR1447 and UniPR1449**

Based on the context of an ELISA binding experiment to evaluate the efficiency of compounds in disrupting the EphA2-ephrin-A1 interaction, it was observed a significant difference in $IC_{50}$ values and the values to inhibit EphA2-ephrinA1 in the U251 glioblastoma cells. Especially the fact that those drugs with an $IC_{50}$ of <10 μM suppressed EphA2 phosphorylation in U251 cells effectively meant that they did so at concentrations about three times higher than the $IC_{50}$ values established in the binding assay.

This observation, in turn, triggered us to raise the hypothesis that reduced efficacy of UniPR1447 and UniPR1449 in cellular assays may be related to precipitation induced by relatively high concentrations of calcium ions. Indeed, literature data quite obviously support such a hypothesis, since it was reported that $Ca^{++}$, being a bivalent cation, is able to induce bile acid derivatives precipitation, thereby reducing the availability of these compounds in the cellular medium[109].

To explore this hypothesis further, we performed dose-response experiments to measure the displacement of ephrin-A1 from its EphA2 receptor using various binding buffers:

- PBS
- Physiological saline solution with $Ca^{++}$ at 2 mM and 4 mM
- Ham-F12 cell medium (Calcium chloride Dyhydrate 44mg/L)
- MEM- minimal essential medium (Calcium chloride Dyhydrate 265mg/L)
- Calcium free physiological saline

Attempts were made to establish if the availability and efficacy of UniPR1447 and UniPR1449 independently in binding assays were affected by the presence of $Ca^{++}$ or any of the components in the cell culture media.

These results further support that the availability and therefore active concentration of UniPR1447 and UniPR1449 in cellular assays are significantly reduced by the presence of $Ca^{++}$ and some components of the cell culture medium. Thus, data suggest that this discrepancy in the $IC_{50}$ values between the ELISA binding assays and the cellular assays would be due to the precipitation of these compounds in the presence of $Ca^{++}$.

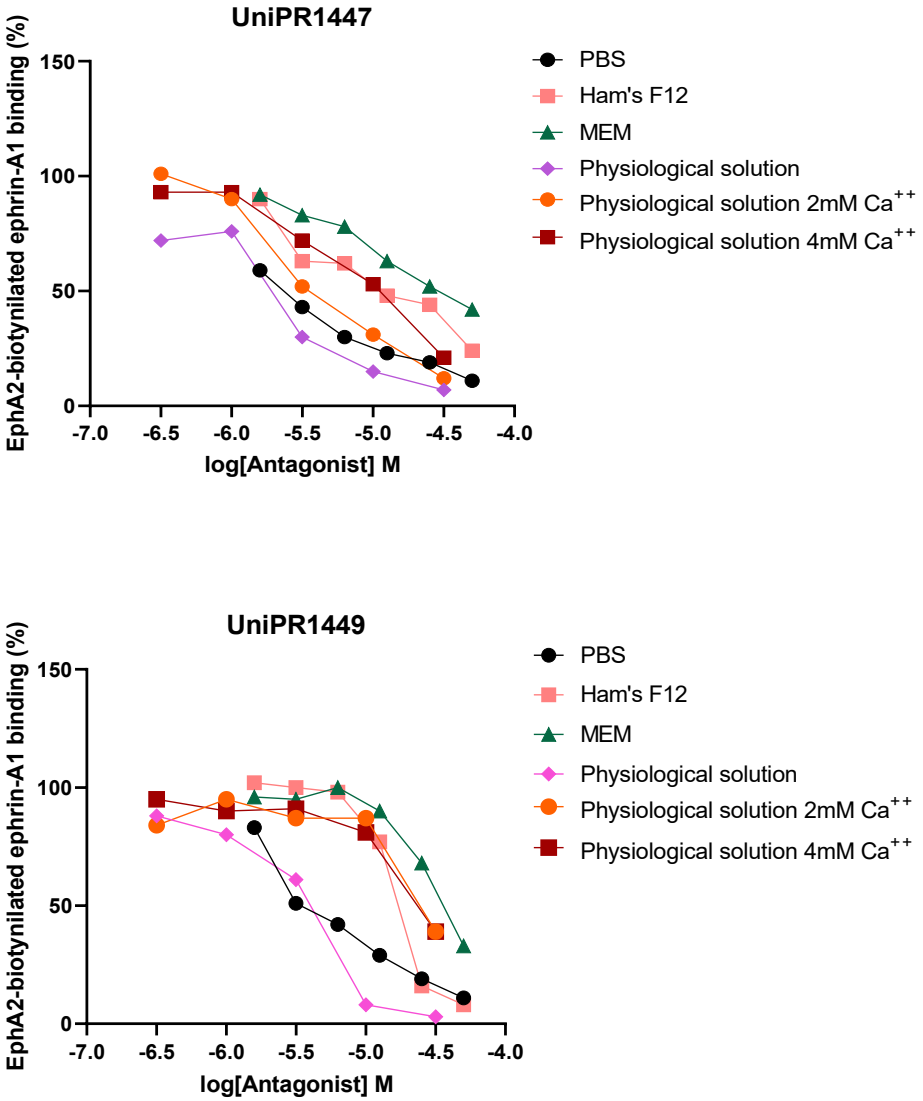



**Fig.26** *Binding curves for UniPR1447 and UniPR1449 in the displacement assay of biotinylated ephrin-A1-Fc from EphA2-Fc. Compounds at the indicated concentrations and 0.5% DMSO (used as control) were added in the wells. Then they were incubated for 1 h at 37 °C. Biotinylated ephrin-A1-Fc was added to the wells and further incubated for an additional 4 h at 37 °C.*

- **Inhibition evaluation of *in vitro* angiogenesis**

The vascular endothelial growth factor family appears to be crucial for tumor-induced neovascularization and includes six subgroups: VEGFA-E and placental growth factor. VEGFA is considered to be the main factor responsible for angiogenesis and differentially acts through the binding with three different VEGFRs expressed on ECs, among which VEGFR2 represents the primary pro-angiogenic receptor. By binding with VEGFR2, VEGF induces dimerization, internalization, and activation of a signal regulating the pro-angiogenic activity of ECs and neovascularization. This pro-angiogenic activity of VEGF/VEGFR2 is further modulated through interactions with other receptor systems, including integrins, neuropilin, and the erythropoietin-producing hepatocellular carcinoma receptor Eph/ephrin system[110].

A previous study, in support of the information given, had sought to describe the behaviour of UNIPR1331 in the inhibition of the angiogenic pathway supported by VEGFR/VEGFR2, by the research group of Prof. M Rusnati. The study found that UniPR1331 was able to bind to VEGFR2, with an $IC_{50}$ of 16 μM. In addition, it was able to inhibit in a dose-dependent manner the VEGF-dependent autophosphorylation of VEGFR2 in HUVECs. Moreover, it showed the ability to block the angiogenic process *in vivo* on zebrafish. Finally, UniPR1331 did not affect the activation of this receptor by its natural ligand FGF2[106].

Based on the correlation between Eph/ephrin and VEGF/VEGFR system, additional studies were performed in the presence of UniPR1447 and UniPR1449 using an *in vitro* model.

The study in an *in vitro* model, named KDR (VEGFR2) Human RTK Kinase Cell Based Antagonist Activity LeadHunter Assay, was carried out by Eurofins Discovery.

As reported in the graph below, fig.27, that UniPR1447 and UniPR1449 are able to inhibit the binding between VEGFR2 and VEGF dose dependently. Indeed, at the concentration of 30μM, they show the maximum inhibiting effect. This could be indicative of their capability for interfering with the given binding pair.

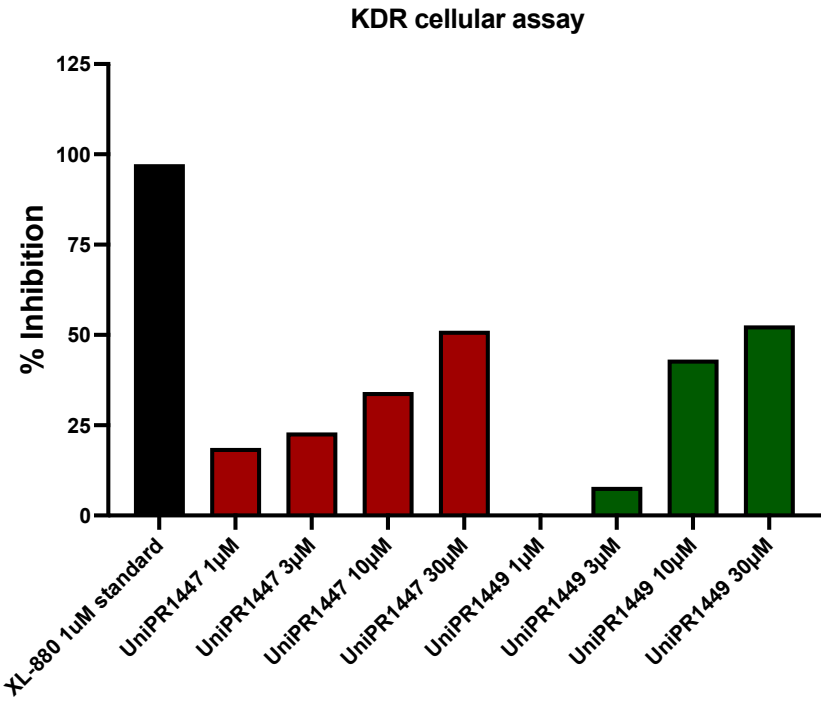



***Fig.27*** *Graph reporting the % of inhibition of VEGFR2-VEGF in HUVECs cell line model.*

- **Characterization of UniPR1447 and UniPR1449 on a model of stem cells**

Following these assays and considering the critical role of stem cells in glioblastoma pathogenesis, I evaluated the behaviour of the two compounds in this context. Thanks to the collaboration with Professor Simona Parrinello's laboratory at the Cancer Institute in London, I conducted several assays to assess the antiproliferative activity of UniPR1447 and UniPR1449 on a glioma stem cell model.

First, I chose a glioblastoma stem cell line: L-11. As shown in the heat map attached (fig.28 A), this cell line expressed overexpression of the ephrin system. More specifically, high expression of the EphA2 receptor was indicated. Thereafter, I performed an immunofluorescence assay on a mouse brain slice previously inoculated with this cell line. This was an important step in verifying that the isolated cell line was a stem cell line. The results of the immunofluorescence (fig.28 B) were positive for two stem cell markers: Sox2 (transcription factor associated with the maintenance of the undifferentiated state of cancer stem cells) and Nestin (neuroepithelial stem cell protein associated with self-renewal/proliferation, differentiation and migration)[25].

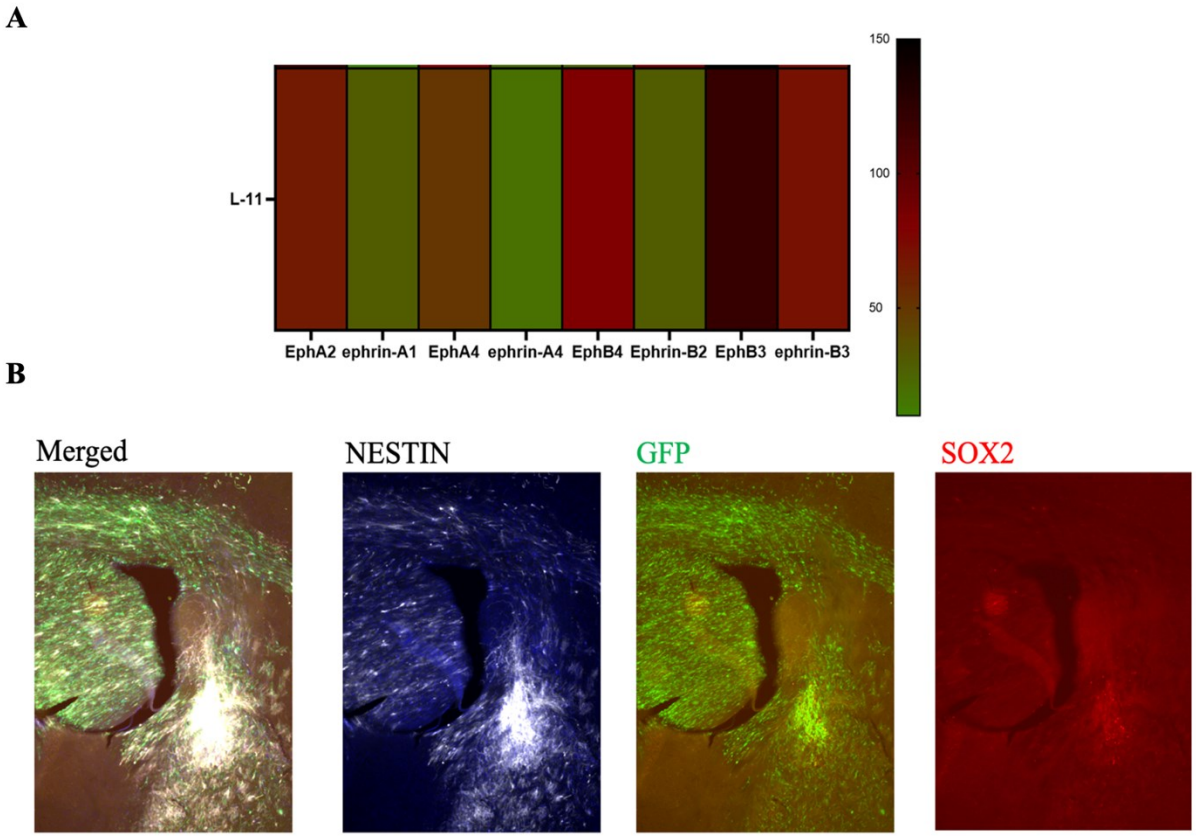



***Fig.28*** *Heat map showing the levels of expression for some Eph receptors and their ligands in L-11 cell line (A). Images relative to expression of stem markers in L-11 cell line (B).*

The data from both the heat map and immunofluorescence assays, taken together, further support the selection of the L-11 glioblastoma stem cell line for further studies. The high expression of EphA2/EphA4/EphB3/EphB4 markers of stem cells indicated that L-11 is the best model for evaluating the activity of UniPR1447 and UniPR1449 against glioblastoma stem cells. This provides a basis for further evaluation of the antiproliferative activities of the compounds against this cell line.

- **Antiproliferative and Cytostatic Evaluation of UniPR1447 and UniPR1449 on Glioblastoma Stem Cells**

To assess the antiproliferative and/or cytostatic effects of UniPR1447 and UniPR1449, the compounds were incubated with L-11 glioblastoma stem cells over various time periods (24, 48, and 72 hours). An MTT assay was employed to quantify cell viability and proliferation.

As reported in the column graphs (fig.29) after 72 Hours of incubation both UniPR1447 and UniPR1449 at 30µM and 10 µM resulted in a marked decrease in cell proliferation. UniPR1449 was also effective at lower concentrations: 3 µM and 1µM.

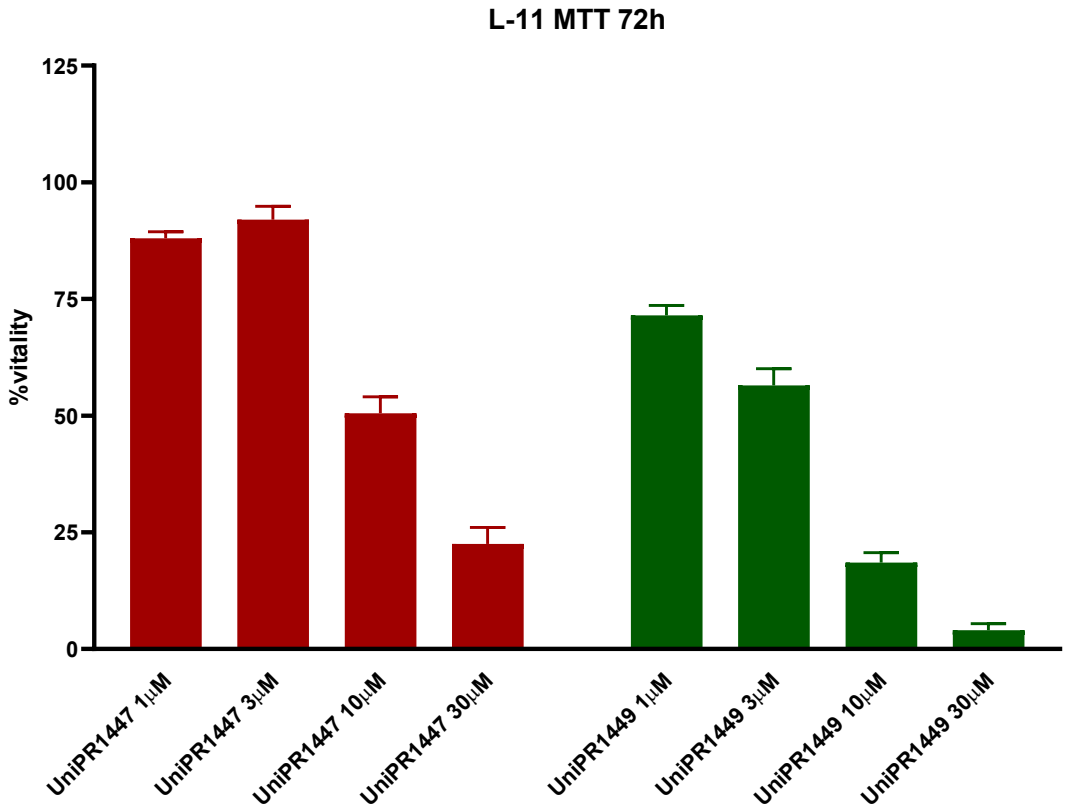



***Fig.29*** *Proliferation of L-11 in presence of different concentrations of compounds. The assay was conducted at 24,48 (data not shown) and 72h with a range concentration from 1 to 30 µM.*

Growth curves (fig.30) were constructed based on the MTT assay data, with the concentration of 30 µM excluded due to excessive toxicity.

Graphs show that UniPR1447 exerts a cytostatic effect at 10 µM in the form of a plateau in the growth curve. That is, UniPR1447 efficiently inhibits the proliferation of cells but does not induce cell death to any appreciable extent at this concentration.

In contrast, UniPR1449 at 10 µM showed a cytotoxic effect after 72 hours of incubation, evidenced by the significant decrease in the number of cells. This result could suggest that the mechanism of action of UniPR1449 at this concentration is not related just to the block of cell proliferation but also to cell death induction. Moreover, it exerts a cytostatic effect at 3 µM and 1 µM since its growth curves underline that there is a remarkable reduction in proliferation in the absence of significant cell death. This suggests that at low concentrations, UniPR1449 efficiently inhibits the process of cell division but not cell death.

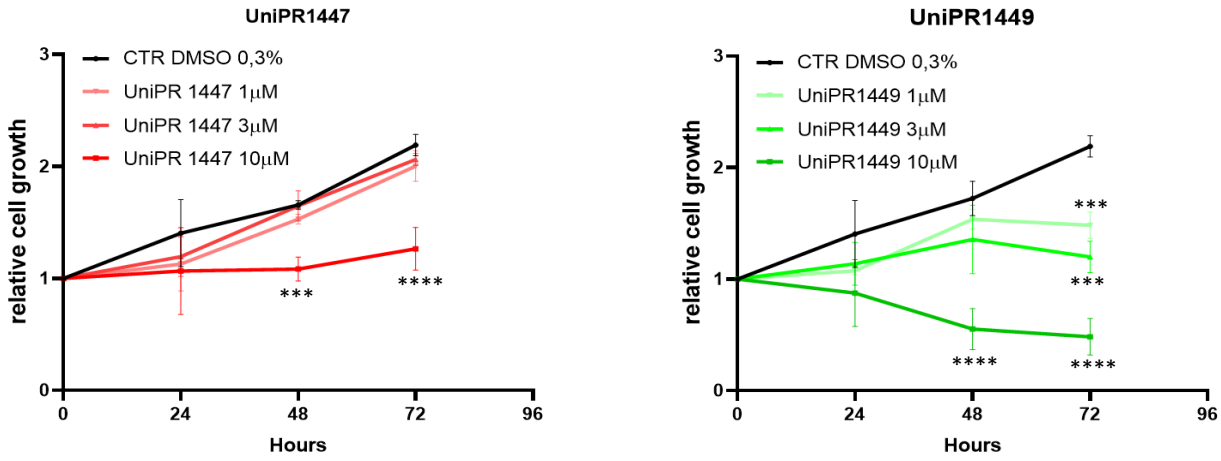



***Fig.30*** *Growth curves of L-11 with and without the compounds. One-way ANOVA followed by Dunnet post-test was performed to compare control curve to all the other curves. *p < 0.05, **p < 0.01, *** p<0.001, **** p<0.0001. Data are the means of at least 3 independent experiments ± SD.*

- **Flow cytometry: antiproliferative action of UniPR1447 and UniPR1449 on glioma stem cells**

The MTT assay, commonly used to assess cell metabolic activity and viability, has certain limitations when applied to glioma stem cells (GSCs). One of the main important limitations is the variability in Metabolic Activity. GSCs can have varying metabolic rates, which might not correlate directly with cell viability. This can lead to inaccurate estimations of cell viability based solely on metabolic activity and that is one of the reasons the MTT assay in the GSCs model is not the best suitable assay.[111]

I used, at this point, a flow cytometry-based method, the 5-ethynyl-2'-deoxyuridine (EdU) incorporation assay, to study in detail the antiproliferative activity of UniPR1447 and UniPR1449 against GSCs. This technique gave an accurate measure of cell proliferation by directly assessing DNA synthesis and provided a very direct way to evaluate cell proliferation, which is very important in assessing the impact of these compounds on cancer cell growth.

GSCs were treated with UniPR1447 and UniPR1449 at concentrations of 10 μM and 3 μM. Such concentrations were chosen according to preliminary MTT assays, which indicated effective doses that are significantly inhibiting cell proliferation without excess toxicity. The cells were incubated with the compounds for 24, 48, and 72 hours.

After incubation, the cells were treated according to the EdU detection kit protocol: fixation, membrane permeabilization, and staining for EdU incorporation.
The percentage of cell cycle distribution in G0-G1, S and G2-phase, which gives a measure of DNA synthesis and hence cell proliferation, was quantified by flow cytometry.
As shown in Fig.31, both compounds demonstrated a dose-dependent decrease in the percentage of cells present in the S-phase after 72 hours of treatment. The higher concentration, 10 μM, had a greater effect, which agreed with the dose-dependent nature of the response.

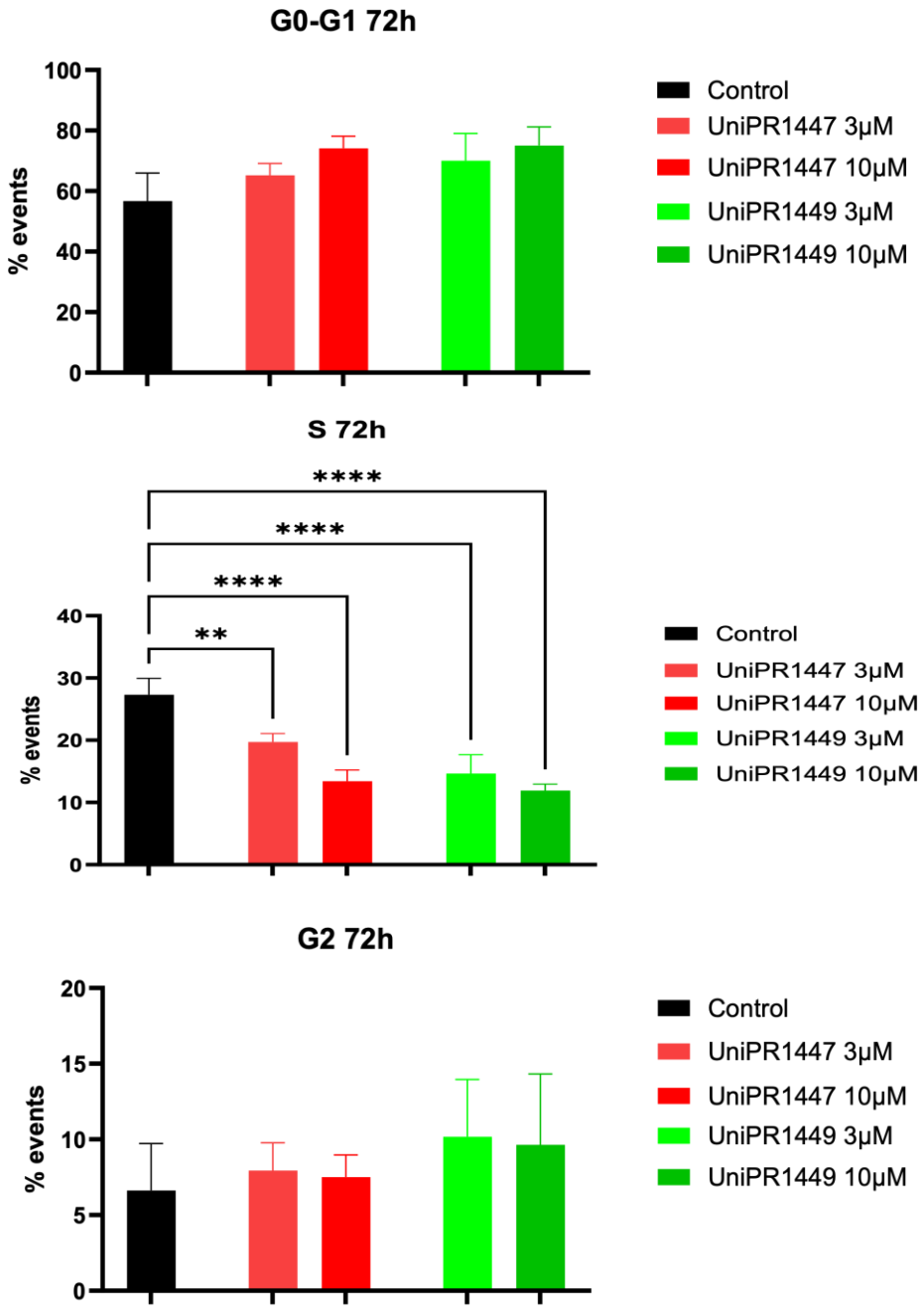



***Fig.31*** *Graph relative to cell cycle results in L-11 cell line in presence of different concentrations of compounds. The assay was conducted at 24,48 (data not shown) and 72h with a concentration range from 3 to 10 μM. One-way ANOVA followed by Dunnet post-test was performed to compare control curve to all the other curves. *p < 0.05, **p < 0.01, *** p<0.001, **** p<0.0001. Data are the means of at least 3 independent experiments ± SD.*

UniPR1449 showed a slightly higher antiproliferative effect than UniPR1447, particularly at 10 μM. This might imply that the structural changes in UniPR1449 are responsible for more effective GSC proliferation inhibitory activity.

The EdU incorporation assay combined with flow cytometry enabled a very potent and highly detailed determination of compound action on DNA synthesis. This methodology provided the confirmation of antiproliferative activity of UniPR1447 and UniPR1449 but also allowed comparative analysis of their efficacy.

- **Evaluation of bioavailability in CD1 male mice following intra-peritoneal administration**

The intra-peritoneal administration of the compounds UniPR1331, UniPR1447, and UniPR1449 was performed in CD1 male mice at a dose of 30 mg/kg. Blood samples were collected by cardiac puncture over a time course of 0-1440 minutes. High-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) was used to determine the plasma concentrations of the compounds. The current study was carried out in collaboration with the research group of Prof. Federica Vacondio of the Food and Drug Area, University of Parma.

Traditional formulation (fine suspension in methocel) of UniPR1331, UniPR1447 and UniPR1449 and one innovative formulation of UniPR1331 were tested. The innovative formulation was the nanoformulation of UniPR1331. This represents a strategy developed by the research group of Prof. Sonvico aimed at enhancing the solubility and bioavailability of the compound.

As can be observed in fig.32, the classical formulations of UniPR1331, UniPR1447, and UniPR1449 possessed a very far from optimal pharmacokinetic profile; hence, these were impractical due to low bioavailability. In contrast, new formulations demonstrated more promising pharmacokinetic profiles, especially concerning AUC, which expresses the overall exposure of the organism to the compound.

**UniPR1331 Nanoformulation**: Among all the formulations tested, nanoformulated UniPR1331 was the most bioavailable. It achieved a $C_{MAX}$ of 2489 nM and an AUC of 855.780μM*min. This represents a great improvement from the traditional formulation.

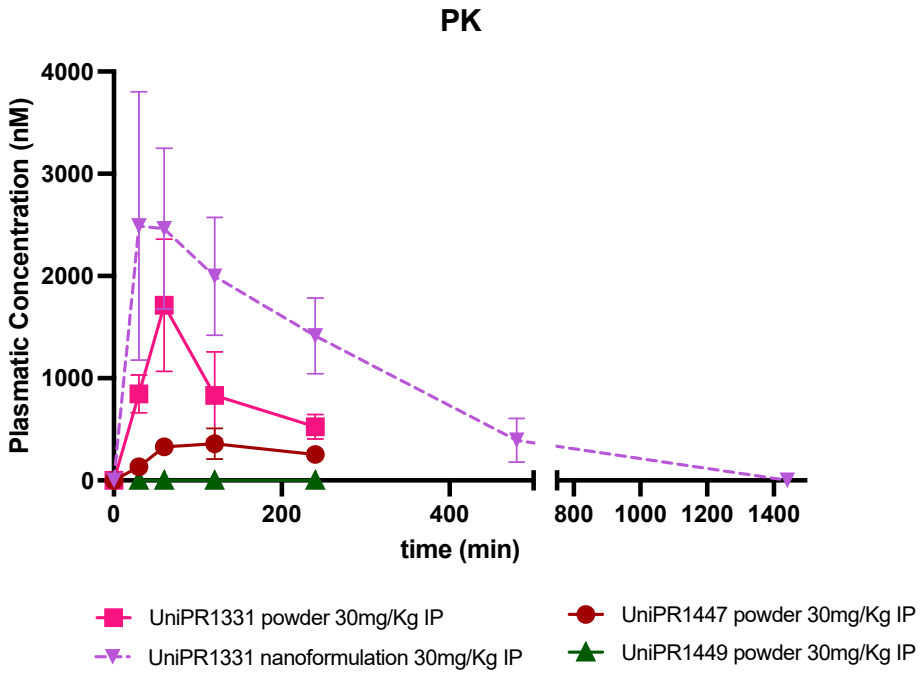



***Fig.32*** *Pharmacokinetic curves: Plasma concentrations (nM) of the compounds over 1440 minutes time course after a single intraperitoneal administration in mice (30 mg/kg).*

The promising pharmacokinetic profiles seen for the innovative formulations, especially nanoformulated UniPR1331, highlight the potential of nanoformulation strategies to enhance bioavailability. The logical follow-through with this research will involve the application of similar nanoformulation techniques to UniPR1447 and UniPR1449. Improvement in solubility and bioavailability should result in significant enhancement of the therapeutic efficacy, rendering these compounds more viable candidates for further development application.

# DISCUSSION and CONCLUSION

Currently, Temozolomide, a chemotherapeutic agent, represents the first line of treatment for glioblastoma. Temozolomide works through a non-specific action to glioblastoma because it alkylates DNA. Temozolomide is a prodrug easily absorbed in the small intestine after oral administration and has strong blood-brain barrier penetration. After administration, it hydrolyses spontaneously inside cells to produce the metabolite responsible of alkylation: MTIC[112].

The use of this drug presents several disadvantages. As a DNA alkylating agent, first, it has low specificity for tumour cells and affects normal cells as well, leading to severe side effects in the patients. The second major limitation, which was mentioned in the introduction, is the presence of cancer stem cells within the glioblastoma population. These stem cells can be quiescent, not necessarily replicating, for extended periods of time. Thus, a molecule that intercalates into the DNA of replicating cells, such as temozolomide, would be ineffective against these non-replicating cancer stem cells[42,113].

Moreover, given the biology of the tumour, glioblastoma is well documented to be highly vascularized, inducing new blood vessels by neo proliferation, which supports tumour growth. This characteristic led to attempts at using another approach: the monoclonal antibody Bevacizumab. The mechanism of action is mediated by targeting and binding VEGF, thereby inhibiting its interaction with VEGFR. Nevertheless, bevacizumab has not produced encouraging outcomes in the treatment of glioblastoma, since it does not prolong the survival of such patients, nor does it prevent serious adverse side effects, including haemorrhages and thrombosis. Bevacizumab is still used but as a second-line therapy for cases of recurrent glioblastoma (CT Number: 2020-003545-11).

Therefore, there is an increase in the need for the exploration of new molecules with specific targets for the future development of therapies that are more focused, ensuring that side effects are minimal, and treatment efficacy is high.

In this context, the Eph-ephrin system comes into play. The Eph-ephrin system is very important in tumorigenesis, and recently, increasing evidence has been confirming dysregulation of ephrins and Eph receptors across tumours[108]. Most of the time, this changed expression level is related to poor patient prognosis, metastasis development, and tumour angiogenesis. In this regard, targeting Eph receptors and their cognate ligands appears to provide a potentially useful means of developing new anti-cancer treatments.
In the introduction I highlighted several different ways this system might be interfered with:
Dasatinib represents a tyrosine kinase inhibitor that proves useful in the treatment of glioblastoma since Eph receptors represent tyrosine kinase receptors. Dasatinib was able to inhibit EphA2 receptor autophosphorylation, therefore reducing receptor activity. But this compound lacks selectivity for Eph receptors and so is precluded from clinical use[86].
Whereas historically there has been no inhibitor targeting the kinase domain of EphA2, more recent developments like GLPG1790 and ALW-II-41-27 have emerged as nanomolar inhibitors of EphA2 with further potential to develop for much-needed effective cancer treatments[81].

It would be helpful the using of antibodies. Monoclonal antibodies against EphA3 were generated to achieve high specificity against the GBM cells. Bispecific antibodies against both EphA2 and EphA3 have shown potential in inhibiting the proliferation of tumour cells[72]. Immunoconjugates such as MEDI-547, generated by linking anti-EphA2 antibodies to cytotoxic agents, are effective in preclinical trials. DS8895a is an afucosylated monoclonal antibody targeting EphA2 and has shown the inhibition of tumour growth in both breast and gastric cancer xenograft models[94].

Other strategies involve the use of peptides, like YSA and SWL. Both are developed to act against the Eph receptors so that the growth of the tumour cells is at a minimum. SNEW is one such peptide, which has been known to inhibit the signaling of EphB2 selectively[95].
The bicycle peptides- of which BT5528 is one example- impede tumour growth in several cancer models. Currently, BT5528 is in clinical trials for use on solid tumors that bear the expression of EphA2[99].

Recent results have been achieved in the development of CAR-T and Epitopes. CAR-T: genetically modified T cells with expression of chimeric antigen receptors against the EphA2 epitopes showed increased survival in preclinical models of glioblastoma. Epitopes: Protein fragments including EphA2 T cell epitopes have been designed and prepared for vaccination, which induces a cytotoxic T cell response against tumors[104,105].

The design of small inhibiting molecules targeting the interaction between Eph receptors, and their natural ligands may thus form a very important approach to such disorders. This is because, as it was introduced, the Eph-ephrin system is overexpressed in a variety of tumors, including glioblastoma. Besides, this system maintains the growth and undifferentiated state of stem cells. Finally, it can also be highlighted that there is crosstalk between the Eph-ephrin system and the angiogenic process through the participation of VEGFR2. For this reason, the development of molecules that target this system could open new avenues for therapies that are more specific[115].

For these reasons, my research over the past three years has focused on characterizing two new small molecules synthesized from UniPR1331.Past studies have demonstrated that UniPR1331 efficiently disrupts the interaction between EphA2 and ephrin-A1; this mechanism of action has been validated by a reduction in tumour growth in an *in vivo* experimental model[103].

However, UniPR1331 is a pan-inhibitor of the receptors, and as previously mentioned, much remains to be discovered regarding the extent to which the two subfamilies A and B are involved in various tumorigenic mechanisms. Therefore, the utilisation and characterisation of two molecules with distinct selectivity profiles for these receptor families may represent a starting point for elucidating these mechanisms. In this context, two novel molecules were designed by the research group of Professor A. Lodola, namely UniPR1447 and UniPR1449. Finally, UniPR1447 is characterised as the β-homologue of UniPR1331 and is described as its L-β-homotryptophan conjugate with 3-β-hydroxy-Δ5-cholenic acid, while UniPR1449 is identified as the N-sulfonylphenyl derivative of UniPR1447[107].

The calculated $IC_{50}$ and Ki values show a good binding affinity of UniPR1447 against EphA2, which interacts with the receptor in a competitive and reversible mode. This can be observed from the saturation curve obtained. Indeed, ephrin, even when used at increasing concentrations

in the presence of the compound, is still able to bind to the receptor. By examining the curve, we see a rightward shift in the binding curve, but even at the highest compound concentration (30 μM), ephrin-A1 is still able to bind to the EphA2 receptor. To further explore the behaviour of binding, I conducted a reversibility assay. As shown in the previously presented graph UniPR1447 turned out being reversible binders for EphA2 receptor, as demonstrated by the restored biotinylated ephrin-A1 binding in wells after inhibitor wash out.

In parallel, however, UniPR1447, like UniPR1331, is a poor discriminator between the two Eph receptor subfamilies. In fact, it demonstrates the ability to bind both EphA2 and various receptor-ligand pairs belonging to the B family. UniPR1447 functions as an antagonist of EphA2 in U251 glioblastoma cells by inhibiting ephrin-A1-mediated receptor phosphorylation. Additionally, it inhibits both the degradation and internalisation of the EphA2 receptor. These three assays confirm that the compound acts as an antagonist of the EphA2 receptor.

Regarding its ability to inhibit the proliferation of U251 GBM cells, we can state that the compound has shown antiproliferative activity without causing non-specific cytotoxic effects. These findings are supported by the MTT and LDH assays. Moreover, based on the growth curves obtained from the MTT data, we can hypothesise a cytostatic effect at a concentration of 30 μM.

UniPR1449 has a strong binding affinity for EphA2, which interacts with the receptor in a competitive and reversible manner, according to the computed $IC_{50}$ and Ki values. This is evident from the obtained saturation curve. Indeed, ephrin can still bind to the receptor even when utilised at higher concentrations when the chemical is present.
Regarding the saturation curve of the EphA2 receptor for UniPR1449, it can be observed that, at a concentration of 30 μM, there is not only a rightward shift but also a decrease in the curve. This result required a reversibility assay to better clarify the nature of the compound's interaction with the receptor. From the data obtained in the reversibility assay, we see that UniPR1449 turned out being reversible binders for EphA2 receptor, as demonstrated by the restored biotinylated ephrin-A1 binding in wells after inhibitor wash out

In contrast to UniPR1447, UniPR1449 shows a preference for the A subfamily of Eph receptors, displaying no binding ability to the receptor-ligand pairs from the B family used in the study.

Similar to UniPR1447, UniPR1449 acts as an antagonist of EphA2 in U251 cells by inhibiting ephrin-A1-induced phosphorylation, preventing receptor degradation and internalization. These results confirmed that, despite structural modifications, UniPR1449 retains its antagonistic nature.

Like UniPR1447, UniPR1449 has demonstrated the ability to inhibit U251 GBM cell proliferation without causing non-specific cytotoxic effects. The growth curve would, therefore, show the cytotoxic effect of UniPR1449 at a concentration of 30 μM and the cytostatic one at 10 μM.

Given the results obtained thanks to the binding studies that allowed me to calculate for the compounds the $IC_{50}$ and Ki values, I conducted a study concerning the specificity of their binding to the LBD site. To this purpose, an assay was performed by Eurofins Discovery. The specificity of drugs for the LBD of EphA2 was investigated by antagonists of the EphA2 kinase domain, UniPR1447 and UniPR1449. It was concluded that they interact with the LBD exclusively; neither of the chemicals interacting with the kinase domain.

The following compounds represent derivatives of UniPR1331, as already stated in the AIM section. This molecule was identified as a direct inhibitor of VEGFR2. Indeed, as described in the Results section, a great deal of research on UniPR1331 and the angiogenic process supported by VEGFR2 was performed by the group of Prof. M. Rusnati at the University of Brescia. It was found from SPR assays that UniPR1331 binds to VEGFR2 at an $IC_{50}$ of 16 μM, showing dose-dependent activity in inhibiting VEGF-dependent autophosphorylation of VEGFR2 in HUVECs. In addition to the *in vitro* activity, it blocked angiogenesis *in vivo* in a zebrafish model without interfering with FGF2-induced angiogenesis, supporting the specificity of its interaction with VEGFR2[106].

Based on the above, I decided to explore the ability for UniPR1447 and UniPR1449 to interfere with the angiogenic process. As more detailed in the Results section, both molecules have been tested in an *in vitro* assay performed by Eurofins Discovery.

Regarding the *in vitro* study, the dose-dependent antagonistic activities of both molecules toward the VEGFR2-VEGF axis have been recorded, quite surely because of their structural

homology with UniPR1331. This represents a promising starting point for further structural modifications in pursuit of enhanced potency while retaining their inhibitory effect on angiogenesis. VEGF-VEGFR2 interaction is critical to the process of the regulation of angiogenesis. VEGF is a specific and potent mitogen for vascular endothelial cells engaged in angiogenesis and vasculogenesis. Its function depends on specific binding to receptors. Two of the VEGF receptors will be VEGFR-Flt-1, or VEGFR-1, and VEGFR-Flk-1/ KDR (VEGFR-2), with the latter playing the key role in all these processes[116].

Moreover, there is evidence of the crosstalk between the Eph-ephrin system and VEGFR2 in the literature. For instance, using the GBM cell lines, it has been shown that EphA2 controls VEGFR2 expression both at the gene and protein level. Inhibition of EphA2 did not affect the expression level of VEGF, indicating that EphA2 may regulate vessel sprouting during developmental angiogenesis independently from VEGFR2 without affecting VEGF levels in GBM cells[117].

Given the well-documented correlation between recurrence of glioblastoma and the presence of GSCs, anti-proliferative activities of UniPR1447 and UniPR1449 were assessed against a GSC model. As can be observed from the results, initially, both compounds were analysed by the MTT test. This test showed that the compounds could inhibit the proliferation of the L-11 cell line at 30 μM and 10 μM after 72 hours of incubation. Furthermore, it is essential to indicate that with compound UniPR1449, such inhibition was also possible to observe at the concentration of 3 μM after 72 hours. Growth curves indicate that compound UniPR1447, at a 10 μM concentration, exerts cytostatic effects. At 10 μM, compound UniPR1449 exhibits clear cytotoxicity, whereas at 3 μM and 1 μM concentrations, it is cytostatic.

As supported by the literature, the MTT assay establishes a correlation between cellular metabolic activity and proliferation. However, when considering glioma stem cells (GSCs), existing studies indicate that metabolic activity and viability have certain limitations. One significant limitation is the variability in metabolic activity; GSCs can exhibit differing metabolic rates, which may not directly correlate with cell viability[111].

For this reason, I analysed the activity of my compounds by examining their potential interactions with the cell cycle, utilising the EdU assay. EdU is a molecule that can bind to the DNA of replicating cells during the S phase. Thus, it is evident that this analysis provides a more accurate assessment of proliferation by excluding metabolic variability.
Moreover, the analysis of the cell cycle in stem cells has been employed in various cancer types for several years. A notable example is the studies involving the molecule Vismodegib, an inhibitor of the Hedgehog pathway. This pathway, similar to the Eph-ephrin axis, is implicated in physiological processes, but its aberrant activation is associated with several cancers, including pancreatic cancer and medulloblastoma[118,119].

Looking at my results, there was a reduction in the number of the cell in the S-phase and increasing the number of cells in the G0/G1 phase.
The decrease of cells in S-phase, it could be interpreted as a positive effect. Namely, during this stage, the majority of DNA replication occurs[120]. This prepares the cell for further division; regarding tumours, this means uncontrolled proliferation. In particular, it is well documented that GSCs have a defective G1 checkpoint[121]. Without this critical check, the cells enter the S-phase and prepare themselves for mitosis. Hence, having compounds which can block the cells in this phase become advantageous for inhibiting their uncontrolled replication. This finding further underscores the importance of analyzing the cell cycle in this type of cells.
As described above, both compounds are UniPR1331 derivatives. The diverse modifications yielded UniPR1449, which displayed a distinct binding selectivity pattern compared to UniPR1447. However, in summary, UniPR1449, as highly reported, does not inhibit some binding pairs in the EphB family. Nevertheless, when the antagonistic action of the two compounds against GBM cells overexpressing EphA2 was assessed, the two compounds presented quite similar activity. Moreover, their potency in inhibiting proliferation turned out to be rather similar within the same cellular model. Indeed, as assessed by MTT assays and by cell cycle alteration in glioblastoma stem cells, both compounds showed closely related efficacy.
This does not conflict with the selectivity of the two compounds shown in the results earlier. In fact, UniPR1449 showed limited binding capability with various components of the EphB family compared with uniPR1447. It has been well documented in literature how the EphA2, where the binding capability of both compounds has been shown, overexpression highly mediates proliferation in GBM cells[122]. With regard to the EphB family, although the functional

role is only partly known, there is no doubt that it plays a part in some mediations, such as migration and invasion, in glioblastoma[69].

In the last, pharmacokinetic of the compounds was evaluated in a CD1 murine model, and as related in the Results section, neither was bioavailable. However, the situation was different in relation to the nanoparticulate formulations of UniPR1331 showing increased plasma bioavailability with respect to the traditional formulation. A result indicating that both compounds shall be tested in the nearest future with nanoparticle formulations in order to enhance their plasma bioavailability.

In this thesis, I have presented the characterization of two molecules that interact with the Eph-ephrin system and have demonstrated different activities when applied to a glioblastoma cell model.

Concluding I want to make some reflections. As underlined so far, the two compounds showed a marked selectivity profile. The UniPR1447 acts as a pan-inhibitor, while UniPR1449 is able to discriminate between the two receptor subfamilies. Such a different behaviour allows me to make the following reflections.

The first is the possible advantage of using selective compounds. Selectivity might represent a starting point in understanding tumorigenic mechanisms supported by the two different receptor families. So far, how the two subfamilies support tumor progression is unknown. Employing a compound selective for either A or B family may permit a better exploration of these mechanisms.

The other advantage of such a selective compound is its likely therapeutic application. As I said during the introduction, while considering pharmacological tools, one could notice that much effort has been made to generate highly selective antibodies directed against various Eph receptors. However, besides this effort, several disadvantages have been related to the use of antibodies. They must, therefore, be administered by slow and extended infusions to avoid their degradation in the stomach. [123].

It is true, as I reported earlier, that bevacizumab, an antibody targeting VEGF, is currently used for the treatment of recurrent glioblastoma (GBM). However, there are certain limitations. The blood–brain barrier (BBB) restricts the penetration of biomacromolecules, including monoclonal antibodies, into brain tumours, despite their high specificity and affinity for their targets[124]. This limitation necessitates higher doses of monoclonal antibodies, which in turn increases off-target toxicity[125]. Moreover, malignant brain tumours can significantly alter the BBB, giving rise to the blood–brain tumour barrier (BBTB), a structure characterized by disorganized and hyperpermeable tumour blood vessels[126] .

In this context, the advantage of having a small molecule with high selectivity might overcome those issues. Moreover, a small molecule that binds to the LBD of a particular receptor could act as a vector for cytotoxic agents, just as it was already done with the antibody, conjugated with Auristatin, directed against EphA2 [127].

On the other hand, I must also emphasize that having a pan-inhibitory behavior compound could confer some therapeutic advantage. Selective inhibition of only one receptor or family is not always a benefit in and of itself. As I have mentioned before, the ephrin system is redundant. However, due to the high promiscuity of the Eph system, the inhibition or downregulation of one family member can be easily bypassed through other Eph receptors or ephrins, reducing the efficacy of the therapy against a specific target[128]. This occurs in numerous pathologies but is particularly true in glioblastoma, where the implication of several receptor subtypes is definitely clear[69].

The last point is that is evident how crucial this system is for developing new targeted molecules in various pathological contexts where the ephrin system plays a non-physiological role.
I must also emphasise that the importance of this complex as a potential target for anti-oncogenic molecules is supported by recent discoveries. As we well know, EphA2 has been characterized both as a tumour suppressor and an oncogene. Ephrin-A1-mediated canonical activation of EphA2 advances its anti-oncogenic capacities: phosphorylation of the intracellular tyrosine kinase space represses the Erk and Akt pathways, which connects with diminished tumour cell development and survival . On the other hand, when unbound, EphA2 gets to be a substrate for serine-threonine kinases (such as Akt, Erk, and PKA), which advance S897 phosphorylation, turning EphA2 into an oncogenic protein. This non-canonical signalling improves cancer movement, attack, metastasis, angiogenesis, and chemo-resistance .
Recently, Shi et al. demonstrated that ligand-free EphA2 undergoes a multimeric assembly related to its addictive features and therapeutic implications. They suggested that inhibiting pro-oncogenic EphA2 non-canonical signalling by disrupting asymmetric Eph-Eph interaction between the LBD and FN2 of adjacent receptors in the same cell membrane may indeed be a rewarding approach[66].
Therefore, the LBD of EphA2 represents a real possibility for intervention with molecules in modulating the pro-oncogenic signalling mediated by EphA2.
These discoveries, therefore, open up future prospects for the development and characterization of molecules that can interact with the Eph-ephrin system. This paves the way for innovative therapeutic approaches, aiming to target this system in various oncogenic contexts, offering potential for more specific and effective treatments.
Based on all the data showed so far, it is clear how it could be interesting in the future to study these molecules in this model to explore better how and where they are able to interfere. In

particular, UniPR1447 and UniPR1449 have shown an antagonist behaviour on the EphA2 receptor, it might be helpful to investigate the modulation of the receptor's pro-oncogenic signalling in their presence.

# KEY FINDINGS FROM THE RESEARCH

- **Binding Affinity & Selectivity**
  - UniPR1447 shows a strong binding affinity for EphA2, acting in a competitive and reversible manner.
  - UniPR1449 also binds to EphA2 in a competitive and reversible manner but shows selectivity for the A subfamily of Eph receptors, unlike UniPR1447, which binds to both EphA2 and various EphB receptor-ligand pairs.
- **Antagonistic Actions**
  - Both compounds act as antagonists of EphA2 in U251 cells, confirmed through inhibition of ephrin-A1-induced phosphorylation and receptor internalization.
- **Antiproliferative Effects**
  - Both UniPR1447 and UniPR1449 show antiproliferative activity in U251 glioblastoma cells.
  - The growth curves based on the MTT assay data indicated that UniPR1447 exerted cytostatic effects at 30 μM, while UniPR1449 had a stronger cytotoxic effect at the same concentration and showed cytostatic effects at 10 μM.
- **Angiogenesis Inhibition**
  - Both UniPR1447 and UniPR1449 were tested for their potential to inhibit angiogenesis, a process critical in tumor growth. They showed dose-dependent antagonism of the VEGF-$VEGFR_2$ axis, a pathway involved in angiogenesis.
- **Effects on GBM Stem Cells**
  - UniPR1447 inhibited GSC proliferation at 30 μM and 10 μM after 72 hours, showing cytostatic effects at 10 μM.

- UniPR1449 inhibited GSC proliferation at 30 μM, 10 μM, and 3 μM after 72 hours, demonstrating cytotoxicity at 30 μM and 10 μM, and cytostatic effects at 3 μM and 1 μM.

- Both compounds caused a reduction in the S-phase (DNA replication) and an increase in the G0/G1 phase(quiescent/rest phase), indicating that they block GSCs from progressing through the cell cycle and inhibit uncontrolled replication.

- **Pharmacokinetics & Bioavailability**

  - Neither UniPR1447 nor UniPR1449 showed bioavailability in a murine model. However, nanoparticle formulations of UniPR1331 demonstrated increased bioavailability, suggesting that similar formulations for UniPR1447 and UniPR1449 could enhance their therapeutic efficacy.

# RESEARCH CONTRIBUTIONS AND OUTPUTS

Poster session

- PHARMACOLOGICAL CHARACTERIZATION OF NEW COMPOUNDS AIMED AT INHIBITING EPH-EPHRIN INTERACTION IN GLIOBLASTOMA.
  *Congresso della Società Italiana di Farmacologia, Roma 15-19/11/2022*

- PHARMACOLOGICAL CHARACTERIZATION OF NEW PP-INHIBITORS DERIVED FROM CHOLENIC ACID AIMED AT INHIBITING EPH-EPHRIN IN DIFFERENT IN VITRO MODELS OF GLIOBLASTOMA.
  *ULLA summer school 2024, Leiden 29-6/7/2024*

Oral Presentation

- UNVEILING POTENTIAL? TARGETING EPH-EPHRIN IN GLIOBLASTOMA STEM CELLS WITH NOVEL PPI-INHIBITORS.
  *Eph-ephrin system congress, Parma 15-16/5/2024*

Publications

- Ephrin receptor type-A2 (EphA2) targeting in cancer: a patent review (2018-present).
  *Tognolini M, Ferrari FR, **Zappia A**, Giorgio C. Expert Opin Ther Pat. 2024 Oct;34(10):1009-1018. doi: 10.1080/13543776.2024.2402382.*

- Discovery of a new 1-(phenylsulfonyl)-1H-indole derivative targeting the EphA2 receptor with antiproliferative activity on U251 glioblastoma cell line.
  *Guidetti L, Castelli R, **Zappia A**, Ferrari FR, Giorgio C, Barocelli E, Pagliaro L, Vento F, Roti G, Scalvini L, Vacondio F, Rivara S, Mor M, Lodola A, Tognolini M. Eur J Med Chem. 2024 Oct 5; 276:116681. doi: 10.1016/j.ejmech.2024.116681.*

- Molecular Determinants of EphA2 and EphB2 Antagonism Enable the Design of Ligands with Improved Selectivity.
  *Guidetti L, **Zappia A**, Scalvini L, Ferrari FR, Giorgio C, Castelli R, Galvani F, Vacondio F, Rivara S, Mor M, Urbinati C, Rusnati M, Tognolini M, Lodola A. J Chem Inf Model. 2023 Nov 13;63(21):6900-6911. doi: 10.1021/acs.jcim.3c01064.*

- Pharmacological characterization of second generation FXR agonists as effective EphA2 antagonists: A successful application of target hopping approach.
  *Ferrari FR, Giorgio C, **Zappia A**, Ballabeni V, Bertoni S, Barocelli E, Scalvini L, Galvani F, Mor M, Lodola A, Tognolini M.Biochem Pharmacol. 2023 Mar; 209:115452. doi: 10.1016/j.bcp.2023.115452.*

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