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The role of Eph receptors in cancer and how to target them: novel approaches in cancer treatment

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Abstract

Introduction: Erythropoietin-producing human hepatocellular (Eph) receptors are among the largest family of tyrosine kinases that are divided into two classes: EphA and EphB receptors. Over the past two decades, their role in cancer has become more evident.

Areas covered: Considering the need for new anticancer treatments and the emerging role of Eph receptors in cancer, this review focuses on molecular mechanisms underlying the pro-tumorigenic effects of Eph receptors that could be exploited for future therapeutic strategies. This review describes the variability in expression levels and different effects on oncogenic and tumour suppressive downstream signalling of Eph receptors in various cancer types, and the small molecules, antibodies and peptides that target these receptors.

Expert opinion: The complexity of Eph signalling is a challenge for the definition of clear targets for cancer treatment. Nevertheless, numerous drugs that target EpHA2 and EpHB4 are currently in clinical trials. However, some Eph targeted drugs also inhibit other tyrosine kinases, so it is unclear to what extent the targeting of Eph receptors contributes to their efficacy. Future research is warranted for an improved understanding of the full network in which Eph receptors function. This will be critical for the improvement of the anticancer effects of drugs that target the Eph receptors.

Keywords: anti-cancer drugs, cancer, Eph receptors, expression levels, receptor tyrosine kinases, cancer, mechanisms.

Article Highlights

- Eph receptors have a unique, bi-directional, mode of signalling that is associated with many different oncogenic and tumour suppressive pathways.
- Eph receptors can have different expression patterns depending on the stage and the type of cancer; understanding these differences is crucial to elucidate the role of Eph receptors and to develop more appropriate drugs.
- Akt is one common down-stream modulator of Eph receptors
- Eph receptors can interact with other tyrosine kinases and intracellular signalling molecules, which can lead to increased Eph expression, or increased cell mobility.
- Only a handful of drugs that target Eph receptors are currently in clinical trials, but many small molecules, peptides and antibodies that target the kinase domain and the ligand binding domain with different affinities seem to have promising properties for clinical use.
- Future research is necessary for an enhanced understanding of the complex network of Eph signalling and the full impact of drugs that target these receptors.

1. Introduction

Erythropoietin-producing human hepatocellular (Eph) receptors are among the largest family of receptor tyrosine kinases (RTKs). It is well known that they play a role in embryonic development and over the past decade their role in disease is becoming more and more clear [1]. There are two subfamilies of Eph receptors, EphA and EphB, which both have a wide variety of implications in disease and especially in cancer [2]. The ligands of the Eph receptors are divided in two subclasses: Ephrin A and Ephrin B ligands. Ephrin ligands are attached to a membrane, therefore binding to a receptor can induce both forward and reverse signalling, a process known as bidirectional signalling [3], [4]. Moreover, different Eph interactions between the same cells can either signal in parallel or in anti-parallel. Through these diverse signalling patterns, Eph signalling can influence a number of cellular progresses, such as cell repulsion, cell-cell adhesion, cell proliferation, tissue boundary information, cell migration or axon guidance [3].

Eph receptors consist of one extracellular domain, which interacts with the different Ephrin ligands, and an intercellular domain. The extracellular domain contains a ligand binding part, which is connected to the transmembrane region through two fibronectin type III regions [2]. The transmembrane region connects the extracellular domain to the kinase domain in the cytoplasm. The kinase domain is attached to a sterile alpha motif (SAM) domain, which can regulate the kinase domain as is observed in the EphA2 receptor [5]. In some cases, Eph receptors can bind a cytoplasmic PDZ domain, which can be important for synaptic signalling [6]. Besides, due to alternative splicing, not all Eph receptors contain a kinase domain and SAM domain. For example, EphA10 and EphB6 lack the kinase and SAM domain and are therefore considered ‘kinase dead’ [7], [8]. The transmembrane region has an important function in Eph signalling. Eph signalling takes place in multiple steps. The process is initiated by the formation of heteromers, or tetramers at high concentrations, by the

extracellular domains of the receptors and their ligands. The heteromers are formed around a hydrophobic loop of the ligand, usually the G-H loop, and then reside in the hydrophobic, ligand-binding pocket of the receptor [9], [10]. As opposed to other RTKs, these structures have to consist of at least two heteromers, consisting of one receptor and one ligand, which combine to form one ring-structured tetramer [10]. This tetramer interacts with both cells and the positioning of the receptor and ligand in this complex make the bidirectional signalling possible [4]. The complex can, for example, activate the kinase region of the receptor through opening of the phosphorylation sites: the serine and tyrosine residues [11]. Often, phosphorylation of these residues can be induced in a ligand-independent fashion as well, which is observed in the EphA2 receptor [12].

The difference between Ephrin A and Ephrin B ligands is the binding of the ligand to the cell surface bound receptor. Ephrin A ligands are attached to the membrane through a glycophosphatidylinositol anchor, while Ephrin B ligands possess a trans-membrane region consisting of a PDZ domain and a cytoplasmic region [2], [6]. The PDZ binding motif of Ephrin B plays an important role in the bi-directional signalling of the Eph/Ephrin system [6]. Besides, the receptors differ in the way the ligands bind to the receptors. EphA receptors barely need to undergo any conformational changes to bind to their ligands, while EphB receptors have to slightly rearrange their ligand-binding site [10]. Nevertheless, both receptors have the ability to form strong structures with their respective ligands, making it possible to let drugs intervene with these interactions [4]. Another region for drug targeting is the kinase domain of the receptors [13], [14]. In general, Ephrin B ligands bind to EphB receptors and EphrinA ligands bind to EphA receptors, with the exception of EphA4, which binds to EphrinB2, and EphB2, which binds to EphrinA5 [3].

In various cancers, the expression of Eph receptors and their ligands is often ambiguous (Table 1) and the associated molecular mechanisms are quite complex [2], [15]–

[17]. Remarkably, Eph receptors can either have a tumour promoter or suppressor function in cancer, which makes it difficult to assess the clinical application of the inhibitors. Nevertheless, efforts have been made to develop drugs, such as JI-101, XL647 and KB004, that target a variety of Eph receptors [18]–[20]. To understand the role and potential clinical use of these, and other inhibitors, the expression levels of the different Eph receptors in cancer are summarized and related to the mechanisms associated with their signalling and difference in expression. For some of the receptors, several potential anti-cancer drugs, both small molecules, antibodies and peptides are discussed. In order to give an overview, the databases enabled by the University of Amsterdam such as PubMed and Elsevier, were searched on the following terms: EphA1-EphA10, EphB1-Ephb6, EphA1-A10 in combination with the term ´cancer´ and EphB1-Ephb6 in combination with the term ´cancer´. For the investigation on clinical intervention, an initial list of drugs was compiled during the investigation of the receptors and then each drug was searched separately in these databases, including clinicalstrials.gov, to investigate the specific properties. No specific inclusive dates were used, but instead the relevance of the papers was judged on their abstract and title.

2. Expression and mechanisms of the EphA receptors

2.1 EphA1

The expression patterns of the Eph receptors are listed in Table 1.

In colorectal cancer and non-melanoma skin cancer, a low expression of the EphA1 receptor was found in metastatic and advanced stages of the disease, while a high expression is associated with locally invasive colorectal cancer [21], [22]. This difference in expression levels was attributed to an inverse correlation between EphA1 expression and methylation; an increased amount of methylation on the CpG islands was indeed detected in later, metastatic

stages of the disease. Similar expression patterns were observed in other Eph receptors, such as EphA2, EphB1, EphB2 and EphB4 [23].

In vitro activation of EphA1 with EphrinA1-fc increased CXCL12 expression, which promotes the chemotaxis, the transition of the endothelial progenitor cells (EPCs) to hepatocellular carcinoma (HCC) cells, and the tube formation of EPCs. These processes promote angiogenesis and tumour growth in HCC [22]. Secretion of CXCL12 was mediated by PI3K/Akt and mTor pathway, suggesting that EphA1 can have an oncogenic effect through activation of this pathway [22].

In contrast, Yamazaki et al. [24] showed that EphA1 receptor activation by EphrinA1 inhibits cell migration, possibly by blocking the kinase activity of integrin-linked kinase, leading to a decrease of Rac-1 and an increased expression of Rho. The Rac1/Rho ratio is important for metastasis: the higher the ratio the higher the chance of metastasis. Hence, this provides a possible mechanism for the tumour-suppressive role of the EphA1 receptor [24].

2.2. EphA2

Similar to EphA1, expression levels of EphA2 differ between different stages of the disease and between different cancers. In non-small cell lung cancer (NSCLC) cell lines, EphA2 is overexpressed in adherent cells. This overexpression is dependent on endothelial growth factor (EGFR) activation; cell adhesion increases EGFR activation, which leads to downstream activation of MP1 and SRC pathways, resulting in an increased transcription of EphA2 mRNA. Binding of integrin to the cell had the same effect, while binding of EphrinA1 induced EphA2 degradation [25]. This is line with the expression pattern of EphA2 in colorectal cancer, where overexpression seemed to be most prevalent in stage 1 and 2 [23].

Similar to other Eph receptors, the EphA2 receptor can be activated through ligand-independent signaling. In glioblastoma cell lines, the overexpression of EphA2 stimulates the tyrosine kinase activity, which activates ERK, and results in serine 897 (S897)

phosphorylation of EphA2 (Figure 1) [12]. This positive feedback loop between ERK and the EphA2 receptor stimulates cell proliferation [12]. *In vivo* stimulation of EphA2-expressing cells with epidermal growth factor (EGF) leads to S897 phosphorylation of EphA2 as well, either through the MEK/ERK/RSK pathway or through the PI3K/Akt pathway [26], [27]. EphA2, however, has a dual relationship with Akt; when phosphorylated at S897, EphA2 is a substrate for Akt, which promotes cell migration and invasion. Inactivation of the receptor through binding of a ligand blocks the interaction with Akt and the promoting effect on migration and cell invasion [26]. This mechanism could explain the oncogenic and tumour suppressive roles of the EphA2 receptor [26]. In nasopharyngeal cell lines, S897 phosphorylated EphA2 activates Akt, through phosphorylation of PI3K. The stimulation of Akt leads to stimulation of Stat3, which promotes the transcription of Sox2 and MYCBP. These proteins then promote the migration and invasion of nasopharyngeal cells (Figure 1) [28]. Lastly, EphA2 promotes invasion in pancreatic cancer cell lines, possibly through phosphorylation of FAK, which is an important modulator of migration [29].

Besides the ligand-induced dephosphorylation of the S897 residue, EphA2 has another tumour suppressive role in lung adenocarcinoma. Binding of EphrinA1-fc to EphA2 inhibits the KRAS/ERK1/2 pathway, which leads to inhibition of proliferation in lung adenocarcinoma cells [30].

Moreover, in lung adenocarcinoma, loss of EphA2 leads to activation of hedgehog signalling, indicating that EphA2 plays a role in inhibiting hedgehog signalling mediated cell proliferation and metastasis [30].

2.3. EphA3

In small cell lung cancer (SCLC) a low expression of EphA3 can contribute to chemo-resistance to various agents. An *in vivo* study in a cisplatin-resistant model showed that upregulation of EphA3 inhibits phosphorylation of PI3K/BMX/STAT3, which reversed

resistance to cisplatin, etoposide and doxorubicin through the promotion of apoptosis [31]. Another study using *in vitro* models of lung cancer showed that activation of EphA3 led to a decrease in phosphorylation of Akt, which inhibited phosphorylation of the transcription factor FOXO3, a downstream effector of Akt. In line with the known role of Akt, this led to an increased level of apoptosis [32].

In radio-resistant head and neck cancer, inhibition of EphA3 with siRNA inhibited the epithelial-to-mesenchymal transition (EMT) possibly through regulating the PTEN/Akt/EMT pathway; knockdown of EphA3 resulted in decreased levels of Akt and increased levels of PTEN [33]. Furthermore, in glioblastoma cells, EphA3 inhibited the MAPK pathway, which kept the cells in an undifferentiated state. Inhibition and internalisation of EphA3 shifted the balance towards differentiated, slowly dividing cells, which are less tumorigenic. The same accounts for EphA2 [34].

In leukaemia cell lines, EphA3 was found to be differentially expressed, and a CpG region was found in the 5´ end of the regulatory region of EphA3; methylation of that island was inversely correlated with EphA3 expression [35].

2.4. EphA4

EphA4 expression is higher in colorectal cancer cells that survived radiation; in particular, the Akt pathway and ERK1/2 pathways seem to be more active in radiation surviving cells as a response to the overexpression of EphA4 [36].

Notably, in gastric cancer, EphA4 expression is correlated with FGFR1 overexpression and is associated with poor survival [37], [38]. Fukai et al. [39] showed that EphA4 formed a complex with FGFR1, which stimulated FGFR1 associated pathways, such as MAPK and Akt. Furthermore, the complex enhanced activities of the proteins Rac1 and CDC42. Activation by Ephrin A1 increased the activities of Rac1 and CDC42 as well. These

downstream effects stimulated the migration and growth of glioblastoma cells *in vitro* (Figure 2) [39]. Besides, silencing of the EphA4 receptor in pancreatic cancer cell lines inhibits the activity of matrix metallopeptidase 2, increases the expression of E-cadherin and decreases the expression of snail, which are all important regulators of cell adhesion and invasion [40]. Therefore, through the effects on these proteins, the EphA4 receptor seems to promote migration [40]. Similarly, in colorectal cancer cell lines, activation of EphA4 reduced E-cadherin levels and disrupted adherent junctions [36]. In contrast, EphA4 had an inhibitory effect on lung cancer cell migration and invasion, possibly through inhibition of ERK1/2 [41]–[43].

2.5. EphA5

In breast cancer cell lines, methylation of the CpG islands at the EphA5 promotor contributed to a decrease in expression, while methylation in patient samples was associated with a higher tumour grade and lymph node metastasis [44]. Similar results were found in prostate cancer and in both cancers the EphA5 receptor could potentially be used as a prognostic marker [44], [45]. A mechanism that could explain the role of EphA5 as a tumour suppressor can be found in hematopoietic stem cells. Nguyen et al. [46] showed that both the interaction between EphA5/EphrinA5 and EphA7/EphrinA5 plays an important role in cell adhesion and migration. Moreover, forward signalling of EphA5 and EphA7 promoted Rac1 gene and protein expression, which can be important for cell migration [46]. The role of this mechanism in cancer, however, has to be further examined.

In lung cancer cells, the high expression of the EphA5 receptor decreased the efficacy of radiation therapy, despite other research pointing out that a high expression is associated with a better prognosis [41], [47]. This effect on radiation therapy can be caused by the stimulating effect that EphA5 has on DNA damage repair systems as silencing of the receptor

caused defect in the G1/S checkpoint, which increased radiosensitivity. These results emphasised the potential of EphA5 as a therapeutic target in lung cancer [47].

2.6. EphA6

In prostate cancer, the overexpression of the EphA6 receptor is correlated with a higher level of lymph node metastasis [48]. In line with this, knockdown of the EphA6 receptor caused a decrease in angiogenesis and cell invasiveness [48]. The receptor might exert these effects through interaction with the PI3K/Akt pathway. Knockdown of EphA6 decreased Akt and the EIF5A2 gene expression, which is a target gene of Akt and a promoter of melanoma cell invasion [48].

2.7. EphA7

In gallbladder adenocarcinoma and glioblastoma, overexpression of EphA7 was associated with metastasis and a poor survival, whereas in lung adenocarcinoma a high expression was associated with a good prognosis [41], [49], [50]. In NSCLC cancer and laryngeal cancer, knockdown of EphA7 resulted in an increased level of apoptosis through up regulation of BAX and caspase-3 and down regulation of anti-apoptotic protein Bcl-2 [51], [52]. In addition, knockdown of EphA7 resulted in higher levels of PTEN and decreased levels of p-Akt, suggesting that the Akt pathway is inhibited through the up regulation of PTEN [51], [52]. These results supported the oncogenic role of EphA7 through stimulation of migration/invasion and inhibition of apoptosis [51], [52].

In prostate cancer and colorectal cancer the down regulation of the EphA7 receptor is due to a hypermethylation on the CpG island [53], [54]. In colorectal cancer this process is partially mediated by SNHG14. SNHG14 suppressed EphA7 expression by increasing the expression of EZH2, a transcription factor that methylates the promoter region of EphA7,

causing the observed downregulation of EphA7 [55]. Another mode of inhibition of EphA7 is observed in osteosarcoma, where miR-448 influences tumour proliferation and migration through targeting the EphA7 receptor. In these tumours, miR-448 was suppressed, causing an overexpression of EphA7, indicating that this inverse relation might have an effect on tumour progression [56]. Li et al. [57] showed that the activation of the EphA7 receptor by, for example, binding of ephrinA5 in prostate cancer can partially suppress tumour progression through the enhancement of apoptosis. This process is facilitated by an increase in BAX levels, activation of caspase-3, reduced Bcl-2 levels and the dephosphorylation of Akt. So, EphA7 has both tumour-suppressive and oncogenic roles through modulation of similar molecules [51], [52], [57].

2.8 EphA8

In epithelial ovarian cancer, overexpression of EphA8 was associated with a higher level of metastasis and this receptor may be useful as a prognostic marker [58]. Similarly, in oral tongue squamous cell carcinoma, EphA8 promotes progression of the tumour. Liu et al. [58] showed that these effects might be due to the effect on invasion of the tumour but not on proliferation. However, more information about the mechanism underlying EphA8 function is warranted.

2.9. EphA10

In breast cancer, cytoplasmic EphA10 stimulates invasion and metastasis and *in vivo* results showed that downregulation of EphA10 led to a better outcome [59]. Furthermore, another membrane-bound isomer of EphA10 promoted β-catenin, which inhibited migration. Down-regulation of this form of EphA10 promoted metastasis [59].

Membrane bound EphA10 receptor exerts no kinase activity as opposed to the other Eph receptors [60]. *In vitro* studies showed that EphA10 exerts its anti-apoptotic effects through inhibition of the EphA7 receptor, which supports the growth of cancer cells [60].

3. Expression and mechanisms of the EphB receptors

3.1. EphB1

In acute myelogenous leukemia (AML) low expression of EphB1 is associated with poor survival, which might be due to the effect of EphB1 on the DNA damage repair system [61]. Reintroduction of EphB1 stimulated expression of several enzymes from the DNA damage repair system, including p53, Chk1, p21, CDK1, Bcl-2 and BAX. After reactivation of Chk1 the AML cells went into G2-M cell cycle arrest [61]. In colorectal cancer, low levels of expression were associated with poorly differentiated tumours, but no methylation of the CpG island was reported [62].

In glioblastoma, ligand-dependent signalling of the EphB1 receptor is a prognostic factor of better outcome [63]. Patient samples showed that the interaction with EphrinB2 is important for inhibition of migration and EphB1 can decrease the EphrinB2 induced migration [63]. In contrast, in medulloblastoma cell lines it was shown that knockdown of EphB1 inhibits migration, pointing out the role of EphB1 as a promoter of migration [64]. Furthermore, in medulloblastoma cell lines, knockdown of EphB1 induced G1 cell cycle arrest and increased radiosensitivity in xenograft mouse models [64]. In contrast, Wei et al. [15] postulated that in a medulloblastoma cell line, ligand-dependent activation of the receptors has an oncogenic effect through multiple downstream molecules, such as cyclin E, PCNA, Akt, Integrin β, Src and EGFR, which have a stimulatory effect on cell growth/viability and migration [15].

3.2. EphB2

Next to the suppression caused by methylation in other receptors, it was found that EphB2 receptor was suppressed in a brain-metastasised prostatic cancer cell line due to a mutation, which inactivated the receptor. Transfection with non-mutated cells led to suppression of tumour growth. Hence, mutations can be a driver of oncogenesis for Eph receptors as well [65]. In pancreatic cancer cells, EphB2 acts as a tumour suppressor through inhibition of proliferation and inhibition of Bcl-2, which promotes apoptosis, whereas in another study a low EphB2 expression in patient samples was correlated with a higher one-year survival rate [66], [67]. Other *in vitro* studies showed that EphB2 is able to induce a non-apoptotic, autophagy-like cell death through regulation of the ERK/MAPK pathway and simultaneous inhibition of Akt [68]. As Akt is a known inhibitor of autophagy, both activation of ERK/MAPK pathway and the inhibition of Akt could independently contribute to this autophagy-like cell death [68].

In both cervical cancer and glioblastoma cell lines, EphB2 overexpression is correlated with r-RAS expression and activation: this promotes EMT and invasiveness [69], [70]. Earlier it was shown that EphB2 activation of r-RAS can interfere with integrin mediated adhesion, while phosphorylation of r-RAS negatively influences “the ability of r-Ras to support integrin activity” [71]. This promoted the migration of renal cancer cells *in vitro* [71]. In medulloblastoma, stimulation of EphB2 by EphrinB1 showed an increase in cell invasion as well [72]. A possible mechanism for this was an observed increase in phosphorylation of P27 and Paxillin [15], [72]. Lastly, the FAK inhibitors PF573228 and 14 could inhibit this increase in invasion, indicating that FAK contributes to the EphB2 induced invasion in glioblastoma cell lines [73].

High EphB2 expression is associated with poor survival in breast cancer patients, but there is an ambiguous role for EphB2 in different carcinogenic stages of breast cancer cells

[74], [75]. To explain this duality, Chukkapelli et al. [75] suggested that EphB2 stimulates autophagy, which, in turn, can either promote invasion or apoptosis based on the context. In a normal situation, autophagy promotes apoptosis, but when apoptosis is blocked in cancer cells, autophagy promotes invasion [75]. This mechanism could explain the ambiguous role of the EphB2 receptor in breast cancer, but future research is necessary to further investigate this theory [75].

3.3 EphB3

Similar to other Eph receptors, EphB3 loses expression in later, metastatic stages of colorectal cancer [76]. *In vitro* studies showed that SNAIL1 and SNAIL2 epigenetically suppress the EphB3 receptor through histone modifications and displacement of other transcription factors; this process could have an important role in the initiation of metastasis [76]. In gastric cancer, EZH2 supresses EphB3 as well through a histone modification, specifically the H3K27me3 [77]. In NSCLC, EphB3 promotes cell growth, migration and survival in a kinase-independent manner [78]. In papillary thyroid cancer knockdown of EphB3 suppressed Rac1 activity and enhanced Rho; the higher the Rac1/Rho ratio, the more metastasis is promoted [79]. In contrast, in gastric cancer, loss of EphB3 promotes EMT, as elevated levels of EphB3 were correlated with increased levels of E-cadherin but decreased levels of vimentin, both important regulators of the EMT [77]. Lastly, in prostate cancer cell lines, high expression levels of EphB3 and EphB4 contributed to local invasion and metastasis [80]. In addition, Li et al. [81] identified a novel pathway through which EphB3 can suppress tumour activity in NSCLC next to its tumour promoter functions. RACK1 forms a complex with phosphorylated EphB3; this complex then forms a tertiary complex with protein phosphatase 2a and Akt (Figure 3). This tertiary complex inhibits the phosphorylation of Akt, resulting in an inhibition of migration [81]. For this process, stimulation by EphrinB1

is necessary. Therefore, since EphB3 is overexpressed in lung cancer and Ephrin B1 is not, formation of the complex is less common [81].

3.4. EphB4

EphB4 has a few downstream effector molecules that contribute to its oncogenic role in some cancers. In ovarian cancer cell lines, the inhibition of EphB4 leads to increased apoptosis, possibly induced by an inhibition of the protein levels of Akt and mTor, which is similar to the role of EphB4 in oesophageal cancer.[82], [83]. Moreover, in prostate cancer EphB4 stimulates integrin beta8, which could promote angiogenesis and in a breast cancer cell line, EphB4 promotes the Ras/c-Raf pathway, possibly through promoting protein phosphatase 2. This could explain the observed promoting effect on migration of EphB4 as well [84]–[86].

Li & Zhao [87] found that EphB4 has a differential expression in pancreatic cancer, as follows: “The EphB4 receptor was significantly higher expressed in stage III and stage IV pancreatic ductal cell cancer compared to stage I and stage II” [87]. In a follow up study, Li et al. [88] showed that down regulation of EphB4 inhibited proliferation and migration of pancreatic cancer cells in vitro. Further research is necessary to assess the role of the EphB4 receptor *in vivo* [88].

In line with research on other Eph receptors, a high expression of EphB4 in colorectal cancer is associated with a higher level of metastasis [89]. Furthermore, notch signalling epigenetically upregulated the EphB4 receptor in colorectal cancer through removing the H3K27me3 on the enhancer region of EphB4 [90]. In gastric cancer, in a hypoxic state, long coding RNA BC005927 upregulated the EphB4 receptor, which had an increased effect on metastasis as well [91].

3.5 EphB6

Similar to the EphA10 receptor, EphB6 has no kinase activity and is thus considered "kinase-dead" [8], [92]. In NSCLC, the silencing is caused by hypermethylation of promoter DNA [93], [94]. In colorectal cancer, low expression of the receptor is associated with a poor prognosis and with a higher level of lymph node metastasis [95]–[98]. In contrast, an overexpression of EphB6 in colorectal cancer had a stimulatory effect on proliferation and invasion. Co-overexpression with the APC regulator of WNT signaling pathway gene might be an important contributor to this effect [99].

In breast cancer, overexpression of EphB6 is associated with a decreased level of invasiveness, which might be due to downstream signalling of EphB6 [100]. Normal expression of EphB6 mediates breast cancer cell adhesive strengths through the phosphorylation of c-Cbl. c-Cbl contributes to the adhesive-stimulating function of EphB6 by activating Abl, a molecule that is important for the stability of the cytoskeleton [101]. Considering the low expression in NSCLC, this might be a possible mechanism through which a lack of EphB6 promotes invasiveness [101]. In triple negative breast cancer, EphB6 has the ability to induce mitochondrial fragmentation through the activation of ERK kinases, which phosphorylate Dynamin-1-like protein (DRP1); the mitochondrial fragmentation makes the cells more susceptible to Death receptor 5 induced apoptosis [7].

Lastly, there is evidence that the EphB6 receptor interferes with EphA2 signalling. EphB6 prevents the anoikis-resistance that is induced by EphA2, while EphB6 prevents the phosphorylation of S897, which is important for ligand-independent oncogenic signalling of EphA2. Through these mechanisms, the EphB6 receptor attenuates the oncogenic signalling of EphA2 [102].

4. Drugs targeting Eph receptors

4.1. Drugs under investigation (Table 2)

4.1.1 Small molecules

Binding domains of the small molecules, peptides and kinase inhibitors are summarised in Table 3, the different drugs either bind to the kinase domain or interrupt interaction with the Ephrin ligands [103].

Noberini et al. [9] identified two isomers from the lead compound 2,5-dimethylpyrrolyl benzoic acid that are capable of binding EphA4. Compound 1 had an IC_{50} of 13 μM and compound 2 of 9 μM for inhibition of the binding of the natural ligand ephrinA5 to the EphA4 receptor in the HT22 neuronal cell line [9]. Besides, the compounds showed a low affinity for the EphA2 receptor. In a follow-up study, binding of these two molecules was confirmed by nuclear magnetic resonance (NMR) titrations. The NMR titrations showed that the binding of the ligand was in the high affinity-binding centre of the EphA4 receptor. However, due to the small size of the molecule, it did not reach other parts of the EphA4 receptor. Therefore, improving the affinity by extending the molecule could be a good direction for future research [104]. Noberini et al. [9] identified a disalicylic acid-furanyl derivative, compound 76D10, with an IC_{50} of 3 μM for inhibition of EphrinA5 binding to EphA4 in the HT22 neuronal cell line. They suggested that both compound 1 and 76D10 might be good lead compounds for the further development of EphA4 antagonists. Inclusion of a salicylic acid group, a known structure of different Eph inhibitors, might be a good strategy for improving antagonists [105]. In a later in vivo study, compound 1 showed efficacy in xenograft mouse models, where it inhibited phosphorylation of both EphA4 and Akt, leading to apoptosis. Furthermore, no major side effects of the drug were found in this mouse model, supporting its potential for further clinical development [106].

Other potential Eph inhibitors are D5-cholenoyl-amino acid derivatives [107]. One derivative, UniPR1331 (compound 10), disrupts the binding of an Ephrin ligand to all Eph

receptors (EphA1-EphA8 & EphB1-EphB6), with IC_{50} values ranging from 2.5 to 5.4 nM [107]. This compound, however does not inhibit kinase activity [107].

NVP-BHG712 has the ability to inhibit EphB4 kinase activity and EphA2 with an IC_{50} of 3 nM in HEK293T cells [108]. Besides, NVP has a high affinity for other Eph receptors as well, with IC50s ranging from 0.3 nM for EphA3 to 303 nM for EphA1. Most of the available NVP derivatives are isomers of the Novartis patented original drug [108]. In general the isomers have a lower affinity, with IC_{50} values of 163 and 1660 nM for EphA2 and EphB4, respectively, which demonstrates that small differences can make a significant impact on Eph receptor binding [108] NVP-BHG712 inhibits VEGFR2 as well, but the drug has a 200 times higher affinity for EphB4 [109]. Furthermore NVP-BHG712 can decrease the ATP binding cassette subfamily C member 10 (ABCC10) mediated efflux of paclitaxel and thus help in overcoming paclitaxel resistance [110]. These results warrant clinical trials, as preclinical research seems promising.

GLPG1790 has an IC_{50} of 11 nM for inhibition of kinase activity of EphA2 in a biochemical assay and an IC_{50} of 260 nM for inhibition of the phosphorylation of the EphA2 receptor in MDA-MB-231 cells [111]. Furthermore, the MAPK pathway was also inhibited, indicating this inhibition as a possible mechanism of action of GLPG1790 [111]. In another study, GLPG1790 reverted the oncogenic phenotype while promoting G1-growth arrest in breast cancer cell lines and xenograft mouse models, which improved radio sensitivity [112]. In a recent study, GLPG1790 showed anti-tumor effects in glioblastoma and clinical trials for glioblastoma are warranted [113].

Doxazosin is an EphA2 agonist with an IC_{50} of 0.74 nM ± 0.30 nM, obtained in MDA-MB-231 cells [13], [114]. In prostate cancer cells Doxazosin inhibited migration and metastasis in vivo, which is in line with the known role of ligands to abrogate EphA2 stimulated migration and invasion [114]. Currently lead optimisation of Doxazosin is on-

going and future research should prove the efficacy of agonists, such as Doxazosin, in patients [13], [114].

4.1.2 Antibodies

Several antibodies target the Eph receptors [115]–[119]. Nagano et al. [118] showed that a monoclonal antibody targeting EphA10 had anti-tumour effects in breast cancers expressing EphA10 [118]. Monoclonal antibodies that target EphA2 are the agonistic antibody IG25, which stimulates the degradation of the EphA2 receptor and the antagonistic antibody IG28, which blocks binding of Ephrin A1 with an IC_{50} value of 0.89 nM in the MC38-CEA colon cancer cell line [119].

Of note, an interesting novel approach is the use of nanobodies. Nanobodies, in general, have a higher affinity for the target and are cheaper to produce than monoclonal antibodies [120]. Schoonaert et al. [103] identified novel nanobodies, Nb39 and Nb53 that target the EphA4 receptor and replaced the binding of all the known Ephrin ligands of the EphA4 receptor with IC_{50} values of 170 and 261 nM, respectively, in an EphA4-expressing E. coli strain [103]. However, more research is needed to define the potential use of nanobodies for the targeting of Eph receptors [103].

4.1.3 Peptides

A third class of drugs that target Eph receptors are peptides [17], [121]. Some of these peptides target the EphA2, EphB4, and EphB2 receptors [121]–[123]. SWL is currently in the pre-clinical phase and has an IC_{50} of 4.1 μM [121]. Duggineni et al. [121] developed a dimer of SWL with an IC_{50} of 0.31 μM, promoting the EphA2 tyrosine phosphorylation in vitro. This agonistic functioning makes SWL an interesting treatment option as opposed to the

other EphA2 antagonists, as stimulation of the receptor could inhibit the ligand-independent, pro-oncogenic signalling observed in the EphA2 receptor.

SNEW is a peptide that targets the EphB2 with an IC_{50} of approximately 15 μM in an EphB2-expressing Baculovirus system [122]. SNEW binds in the hydrophobic binding cleft of the EphB2 receptor, thereby inhibiting the binding of the receptor with EphrinB2. TNYL-RAW is a peptide that disrupts the binding of EphrinB2 with the EphB4 receptor but with a higher efficacy; the IC_{50} value is 15 nM, which was obtained in an EphB4-expressing Baculovirus system . Binding of SNEW could be improved by adjusting the c-terminus as described by Ma et al. [124]. A possibility would be the addition of a PXSPY motif to the c-terminus, which could also be used to improve the binding affinity to the EphB2 receptor.

4.2 Drugs in clinical trial (Table 4)

4.2.1. Dasatinib

Dasatinib is a multi-kinase inhibitor and is used for the treatment of chronic myelogenous leukemia by inhibiting BCR-ABL1, but it is also known to inhibit various Eph receptors. In breast cancer cell lines, both EphA2 expression levels and EphA2 phosphorylation levels are down regulated as a response to dasatinib treatment [125]. In melanoma cell lines dasatinib was not able to induce an effect on EphA2 phosphorylation, indicating that both the cell line and the type of cancer important [126]. Huang et al. [125] demonstrated that dasatinib can inhibit the kinase activity of EphA2 directly with an IC_{50} value of 17 nM in an EphA2-expressing baculovirus system. Next to this direct inhibition, dasatinib might induce down regulation of EphA2 through inhibition of SRC family kinases (SFK), since SFK can regulate EphA2 expression [125]. Dasatinib can also inhibit EphB4 *in vitro* with an IC_{50} of 5.5 nM [127]. Bantscheff et al. [127] showed that imatinib and bosutinib have an affinity for EphB4 as well with IC_{50} values of 5% at 5 μM and 5.5 nM, respectively.

All three IC50 values were obtained in K562 cells. In pancreatic cancer, dasatinib can inhibit ligand-induced EphA2 internalization as well as degradation of the receptor [128].

Side effects of dasatinib include a low blood count, internal bleeding and fluid retention, leading to easy bruising, fever, weight gain and swelling of body parts [129]. Furthermore, dasatinib can cause headache, diarrhea, and fatigue [129]. However, many clinical trials are ongoing for other indications in different cancers, in which Eph inhibition might play a role.

4.2.2. MGCD516 (Sitravinib)

MGCD516 is a novel tyrosine kinase inhibitor, which targets a wide variety of kinases in sarcoma, including PDFGR (IC_{50} of 30 nM) and VEGFR1 (IC_{50} of 6 nM), but also several Eph receptors: EphA2, EphA3, EphA4, EphB2, EphB4 and EphB3 with IC_{50} values of 44, 1, 76, 10, 12 and 249 nM, respectively, in sarcoma cell lines [130]. In three of the five tested cell lines MGCD516 inhibited proliferation, which is possibly due to the wide range of targets and inhibitory downstream effects on p-Akt [130]. A phase 1 clinical trial in solid tumours showed that treatment with MGCD516 (150 mg/day) had limited side-effects, mostly gastrointestinal related, such as nausea, diarrhoea and decreased appetite [131]. In another phase 1 study in NSCLC, Eph receptors were not used as molecular marker for enrolment in the study, but the selection was based on alterations of target RTKs of MGCD516, namely RET, KDR, PDGFRA, KIT, TRK, DDR2, MET, AXL or by loss of function mutations in CBL [132]. The dose for this clinical trial is 150 mg a day with 21 days per cycle [132]. The contribution of Eph receptor inhibition in these clinical trials has to be further investigated, but it is likely that Eph receptors have a role in the mechanism of action of MGCD516. Multiple phase 1, 2 and 3 trials are currently ongoing to test the efficacy of

MGCD516 in NSCLC, liposarcoma, advanced cancers, squamous cell carcinoma, urothelial carcinoma either as monotherapy or combination therapy [133].

4.2.3. JI-101 and XL647

JI-101 is a multi-tyrosine kinase inhibitor with a high selectivity for EphB4, VEGFR2 and PDGFRβ [134]. In phase 1 and 2 clinical trials, the drug was well tolerated and led to 25% stable disease. The tested doses, 100 mg or 200 mg daily, did not cause severe adverse effects. In some patients, side-effects such as hypertension and proteinuria were observed, but no diarrhoea [135], [136]. In some patients treated with a higher dose (400 mg), short-lasting hand-foot syndrome was also observed [136]. In a pharmacokinetics study, JL-101 showed median progression free survival of 5.6 months in ovarian cancer patients in which mostly gastrointestinal side-effects, such as nausea and abdominal pain were observed [18]. A study in rats showed a good oral availability (55%) and a rapid absorption in the body with an accumulation of the drug in the lungs, liver, kidney and small intestine [137].

Considering the ability to inhibit both angiogenesis and proliferation, combination therapies with mTOR inhibitors, such as everolimus, and PI3K inhibitors are considered[18], [134], [138]. In a small clinical study, no serious adverse side effects were observed when assessing the combination therapy of JI-101 and everolimus [138]. In another study, the most frequent side-effect was hypertension, which is seen in a variety of angiogenesis inhibitors [18]. Both studies warrant further clinical evaluation [18], [138]. Currently no clinical trials are recruiting, but, based on the results, future studies on combination treatments with drugs such as everolimus show promise.

Chen et al. [19] described that XL647 as a potent EphB4 inhibitor. Currently, XL647 is being tested in phase 1/2 clinical trials and has shown an IC_{50} value of 1.4 nM.

4.2.4 KB004 (Ifabotuzumab)

KB004 is an antibody that targets the EphA3 receptor with a K_d of 610 pM [20]. In a phase 1 clinical trial in hematologic malignancies, no major adverse effects were observed. The most common side effects were infusion reactions, such as nausea and back pain [20]. Furthermore, no drug-drug interactions were observed, which is in line with other antibodies. Although not the primary aim of this phase I study, only some patients showed a response to the treatment, which led to the conclusion that KB004 is probably not a suitable drug for hematologic malignancies [20]. Nevertheless, a phase 1 clinical trial is currently ongoing for glioblastoma patients [139].

5. Conclusion

The different expression patterns and mechanisms of Eph receptors and drugs that target them, should be evaluated for the assessment of future clinical intervention. For several receptors, such as the A1 receptor, a differential expression was found in different stages of the disease, while for other receptors this differential expression was dependent on the type of cancer. This difference in expression is often due methylation, but in some receptors this could be attributed to the involvement of other receptors, or to microRNA and mutations. All of the reviewed drugs, except for the antibodies, target either the ligand binding pocket of the receptor or the kinase domain. So, drug development and optimisation based on these domains is currently ongoing and some drugs, such as XL64, are in the clinical trial stage, while other drugs need more research. Nevertheless, based on this review, the targeting of Eph receptors could become a useful tool for cancer treatment in the future.

6. Expert opinion

Eph receptors form a group of complex network signalling, which seem suitable for clinical targeting. There is one common target downstream all Eph receptors, Akt. Akt mediates the pro –or anti-tumorigenic effects on proliferation and migration as seen in the EphA1, EphA2, EphA3, EphA4, EphA6, EphA7, EphB1, EphB2, EphB3 and EphB4, while, depending on the specific receptor and the state of the disease, Eph receptors can have both inhibiting and promoting effects on the phosphorylation of Akt. Apart from Akt, the Eph receptors can influence many other downstream molecules that have an effect on the progression of tumours. Besides, Eph receptors have a differential expression patterns between patients, cell lines, stages of the disease and different cancers. All of these factors could influence the efficacy of the drug, so further evaluation of the complex network of mechanisms and expression patterns is necessary to assess the right use of Eph inhibitors in the clinic.

The differences observed in tumour suppressor and oncogenic functions can be related to ligand dependent and ligand independent signalling, as, for example, observed in the EphB1, EphA2 and EphB3 receptors. Activation by a ligand induced a different response than ligand-independent activation and could therefore influence the role of the receptor in the onset of tumours. Another factor that could play a role is the ability of Eph receptors to influence each other and to influence other tyrosine kinases. The 'kinase dead' receptors EphA10 and EphB6 influenced other EphA receptors and exerted anti-apoptotic effects through inhibition of these receptors, while EphB6 also attenuates the oncogenic signalling of the EphA2 receptor. Both the influence of Eph receptors on each other as well as their interaction with other receptors, such as FGFR and EFGR, should be further evaluated, to aid the understanding of the clinical effect of Eph inhibitors.

Several drugs have been developed that target the Eph systems and that could potentially aid in cancer treatment. Interesting targets are EphA4 and EphB4, since a high

expression of these receptors was found in various cancers. Intervention with, for example, compound 1 could be interesting and future research should focus on developing more EphA4 inhibitors. Drugs that have a (high) affinity for EphB4 include dasatinib, JI-101, XL647 and NVP-BHG712 and most of them seem to be promising preclinical and in early clinical trials. For the drugs discussed, EphA2 is a potential target, although the mechanisms are more complex. EphA2 agonists could be interesting to attenuate oncogenic signalling by targeting the ligand-independent activation of Akt. Possible options for this could be the SWL peptide or Doxazosin, which both target the EphA2 receptor. These methods could also be effective for targeting EphA4, EphA7 or EphB3 in NSCLC as these receptors are highly expressed and appear to be linked to a positive prognosis or a tumour suppressive pathway.

Another field for future research is the effect of Eph-intervening drugs on bidirectional signalling, as the bidirectional signalling could have implications for the effects of drugs that disrupt the binding of Ephrins to the receptor.

Drug development for Eph receptors is progressing in line with the increased insight in interactions and in the mechanisms and expression patterns of Eph receptors. This will create a better understanding of the impact of drugs that intervene with Eph signalling and more drugs will be evaluated in clinical trials. With this increased knowledge, it is likely that Eph receptors will gain a place in the current therapies for cancer treatment.

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Table 1. Expression levels of the Eph receptors in different cancers.

Type of cancer	High expression/ Oncogenic functioning	Low expression/ Tumour-suppressive functioning	Differentially expressed
Colorectal cancer	EphA2 [140], EphA4 [36], EphB4 [141]	EphA3 [142], EphA7 [53],	EphA1 [23], EphB6 [95]*, EphB1 [62]*, EphB2 [143], EphB4 [89] EphB3 [76]
Prostate cancer	EphA6 [48], EphA1 [144]*****	EphA5 [45], EphA7 [54], [57], EphB2 [65]	EphA1 [144]
Hepatocellular carcinoma	EphA1 [22]		
Ovarian cancer	EphA2[145] , EphA8 [58], EphB4 [82]	EphA5 [146]	EphB1 [147]*,
Glioblastoma	EphA2 [27], EphA3 [34], EphA7 [50], EphA4 [39]	EphB1 [63]	
Medulloblastoma	EphB1 [64], EphB2 [72]		
Breast cancer	EphA2 [148], EphA8 [149], EphB2 [75], EphB4 [84]	EphA5 [44], EphB6 [100]	EphA10 [59]
Oesophageal squamous carcinoma	EphA2 [150]*****, EphB4 [83]	EphA7 [151]	
Non small cell lung cancer	EphA7 [41]**, EphA4 [41]**, EphA5 [47]	EphB6 [94]	EphA2 [152]
Small cell lung cancer		EphA3 [31]	
Gastric cancer	EphA3 [153], EphA4 [154], EphA8 [149]	EphB2 [155], EphB3 [77]	EphA7 [156], EphB1 [157]*, EphB6 [98]
Acute pediatric myelogenous leukemia		EphB1 [61]	
Renal cancer		EphA5 [158]***, EphB1 [159]	
Oral tongue squamous carcinoma	EphA8 [160]		
Laryngeal cancer	EphA7 [51]		
Head and Neck cancer	EphB4 [136], EphA3 [33]****		
Papillary thyroid carcinoma	EphB3 [59]		
Cervical cancer	EphB2 [69]		EphA4 [161]*
Pancreatic cancer	EphA1 [162]*****, EphA4 [162]*****, EphA5 [162]*****,		EphB4 [87], EphA2 [29]

	EphA7 [162]*****
Rectal cancer	EphA4 [163]*

*has a low expression in cancers with poor prognostic markers (e.g. poorly differentiated and highly invasive tumours) [62], [95], [157], [161]
**favourable prognoses [41], [43].
***in 61.5% of the specimens investigated [158]
****In radio-resistant head and neck cancer [33].
*****In about 50% of the patient specimens [144], [150], [162]

Table 2. Small molecules and antibodies targeting various EphA and EphB receptors

Drug	Kind of drug	Eph receptor targets	Clinical trial status	Dose	IC_{50}
Dasatinib	Kinase inhibitor	EphA2, EphB4, EphA4	Phase 4		17 nM [125], 5.5 nM [126]
Imatinib	Kinase inhibitor	EphB4	Phase 4		5% at 5 x 10^3 nM [127]
Bosutinib	Small molecule	EphB4	Phase 4		5.5 nM [127]
MGCD516	Small molecule	EphA1, EphA2, EphB1, EphB2, EphB4, EphB3	Phase 1/2/3		44,1, 76 10, 12, 249 nM [130]
JI-101	Small molecule	EphB4	Phase 2		-
KB004	Monoclonal antibody	EphA3	Phase 1		Kd = 610 pM [20]
XL647	Small molecule	EphB4	Phase 2		1.4 nM [19]
Compound 1	Small molecule	EphA4, EphA2	N/A		1.3 x 10^4 nM [9]
Compound 2	Small molecule	EphA4	N/A		9 x 10^3 nM [9]
76D10	Small molecule	EphA4	N/A		3 x 10^3 nM [105]
NVP-BHG712	Small molecule	EphB4, EphA2	N/A		3 nM [108]
UniPR1331	Small molecule	Multiple Eph receptors	N/A		Ranging from 2.5-5.4 nM [107]
GLPG1790	Small molecule	EphA2	N/A		11 nM [111]
Doxazosin	Small molecule	EphA2	N/A		0.74 nM ± 0.30 nM [114]
NB39, NB53	Nano bodies	EphA4	N/A		170 & 261 nM [103]
IGg25, IGg28	Monoclonal antibodies	EphA2	N/A		IGg28 0.89 nM [119]
SWL	Peptide	EphA2	N/A		4100 nM [121]
SWL-dimer	Peptide	EphA2	N/A		310 nM [121]
SNEW	Peptide	EphB2	N/A		1.4 x 10^4 nM [122]
TNYL-RAW	Peptide	EphB4	N/A		15 nM [123]

Table 3. Summary of the binding domains of the different drugs

Drug	Binding domain
Dasatinib	Kinase domain [14]
KB004	Site next to the ligand binding site [164]
XL647	Kinase domain [107]
Compound 1	Ligand binding pocket of the receptor [9]
Compound 2	Ligand binding pocket of the receptor [9]
76D10	Ligand binding pocket of the receptor[105]
NVP-BHG712	Kinase domain [108], [109]
UniPR1331	Ligand binding pocket of the receptor [107]
GLPG1790	Kinase domain [113]
Doxazosin	Ligand binding pocket of the receptor [114]
NB39, NB53	Ligand binding domain [103]
SWL	Ligand binding pocket the receptor [121]
SWL-dimer	Ligand binding pocket of receptor[121]
SNEW	Ligand binding pocket of the receptor [122], [123]
TNYL-RAW	Ligand binding pocket of the receptor [122], [123]

Table 4. Summary of several selected clinical trials

Drug	Study and ref	n	Response	Toxicity	Notes
MGCD516	Phase 1[131]	28		Minor AEs, 1 grade 3 palmar plantar erythrodysesthesia at 80 mg	Multiple trials without mentioning Eph receptors as targets
JI-101	Phase 1[135]	18	25% stable disease	Some grade 3 AEs, like fatigue, hypertension and palmar plantar erythrodysesthesia	Very promising and limited side-effects, but currently no new clinical trials
KB004	Phase 1 [20]	64	Limited response	Mostly short-term grade 1 and grade 2 IRs	Clinical trial for glioblastoma patients ongoing

Figures

Figure 1. Different mechanisms that contribute to the oncogenic functioning of EphA2. A) The overexpression of EphA2 leads to ERK activation, which leads to phosphorylation of the S897 residue, inducing a stimulatory effect on cell migration (adapted from [12]). B) Growth factor binding leads to phosphorylation of EPHA2 on the S897 residue, through AKT and other molecules; this leads to the transcription of Sox2 and cMyc which promotes cell migration and invasion [27], [28].

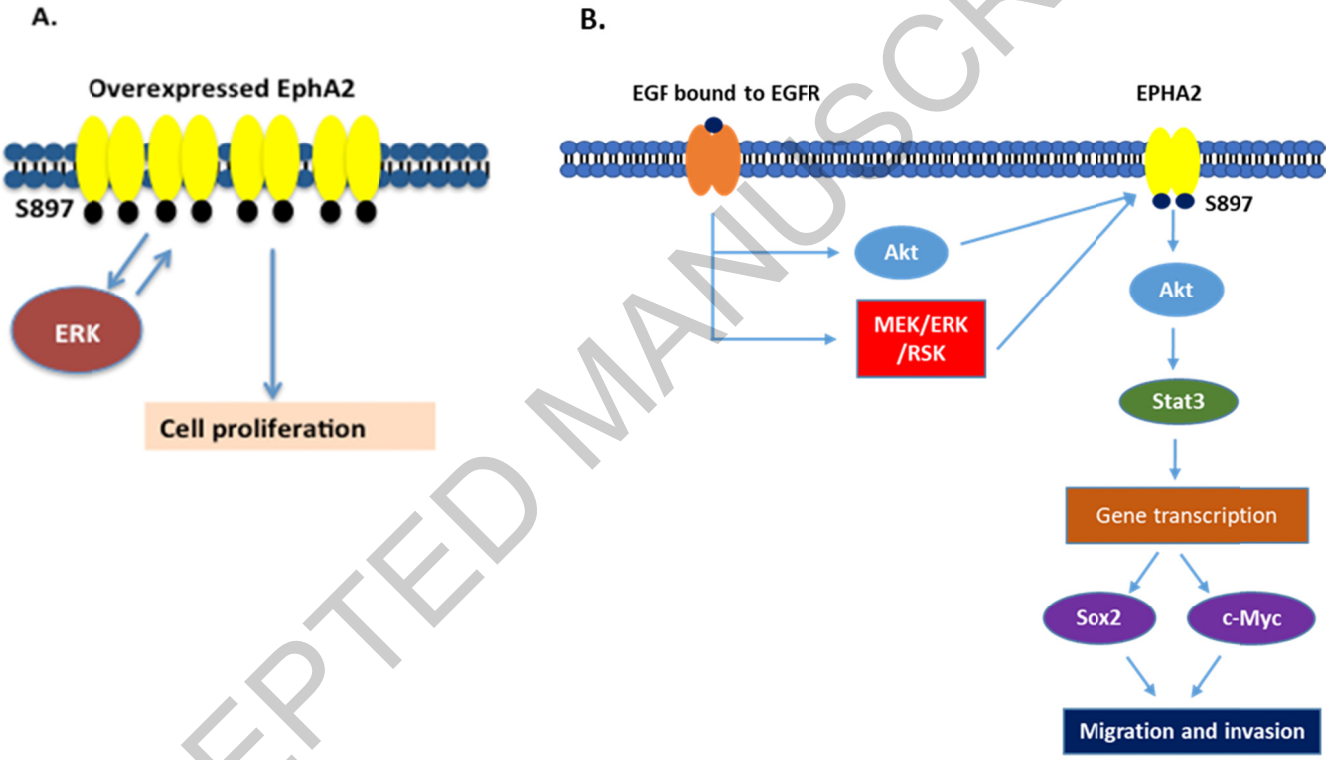

Figure 2. Different mechanisms of the downstream effects of EphA4. Both form a complex with FGFR1 and binding of a ligand, ephrinA1 can induce downstream tumorigenic effects (Adjusted from [81]).

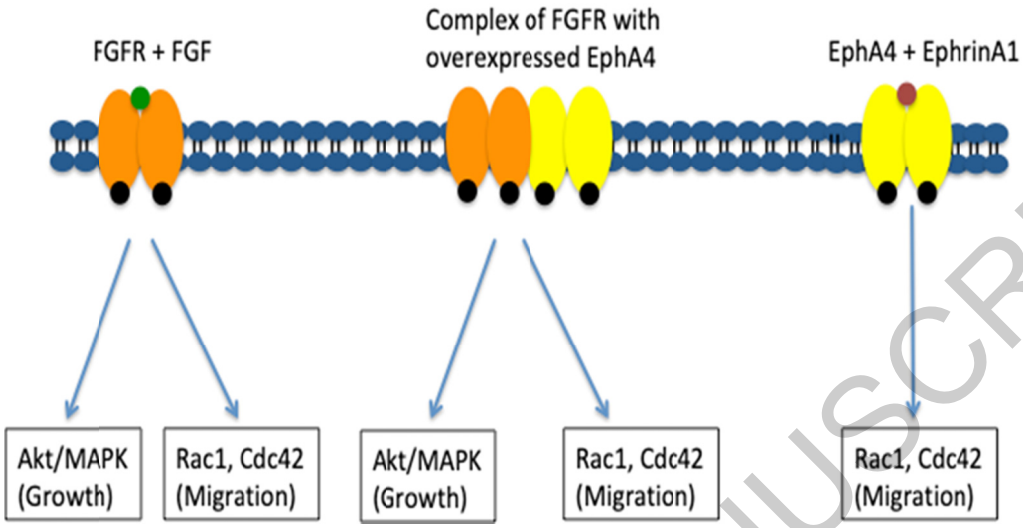

Figure 3. Upon binding with Ephrin B1, EphB3 forms a tertiary complex with Rack1, PP2A and Akt, which prevents the phosphorylation of Akt and subsequent migration (adapted from [81]).