Sprouting strategies and dead ends in anti-angiogenic targeting of NETs

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Abstract

Neuroendocrine tumors (NETs) are a heterogeneous group of neoplasms that arise from cells of the neuroendocrine system. NETs are characterized by being highly vascularized tumors that produce large amounts of proangiogenic factors. Due to their complexity and heterogeneity, progress in the development of successful therapeutic approaches has been limited. For instance, standard chemotherapy-based therapies have proven to be poorly selective for tumor cells and toxic for normal tissues. Considering the urge to develop an efficient therapy to treat NET patients, vascular targeting has been proposed as a new approach to block tumor growth. This review provides an update of the mechanisms regulating different components of vessels and their contribution to tumor progression in order to develop new therapeutic drugs. Following the description of classical anti-angiogenic therapies that target VEGF pathway, new angiogenic targets such as PDGFs, EGFs, FGFs and semaphorins are further explored. Based on recent research in the field, the combination of therapies that target multiple and different components of vessel formation would be the best approach to specifically target NETs and inhibit tumor growth.

Key Words

- ▶ angiogenesis
- ▶ anti-angiogenic therapy
- ▶ new anti-angiogenic drugs
- ▶ neuroendocrine tumors

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Heterogeneity of NETs

Neuroendocrine tumors (NETs) are defined as a heterogeneous group of neoplasms that arise from cells of the neuroendocrine system. Unfortunately, while many of these tumors are benign, some become malignant. Moreover, even though they are described as rare malignancies, the incidence and prevalence of NETs have increased in the last years, and most of them happen to be in advanced stages at the time of diagnosis (Yao *et al.* 2008a).

Since NETs show a wide variety of clinical presentations, different terminology and classifications are used to name the different types of neoplasm. In 2010, the World Health Organization (WHO) updated its classification of NETs based on clinical syndrome, differentiation and site of origin (Obergh & Castellano 2008, Yao *et al.* 2008a, Klimstra 2016).

NETs can be divided into carcinoids or pancreatic NETs. Based on their clinical syndrome, they are characterized histologically by the presence of specific endocrine tissue markers such as chromogranin A, synaptophysin and neuro-specific enolase, or by their ability or inability to produce bioactive peptides and neuroamines, such as serotonin, histamine, prostaglandins, substance P, insulin, gastrin, glucagon and vasoactive intestinal polypeptide (Massironi *et al.* 2008). Related to their pathological grade and state of differentiation and aggressiveness, NETs are divided into three different groups: low grade (G1), intermediate grade (G2) and high-grade tumors (G3). Finally, NETs can be also grouped according to the localization of the tumor and their embryonic origin in the foregut (lungs,

thymus, stomach and duodenum), the midgut (jejunum, ileum, appendix and proximal large bowel) and the hindgut (distal colon and rectum).

As depicted by the complexity of NET classification, the heterogeneity of NETs has further contributed to their limited therapeutic options. Hence, there is an urge to develop an efficient therapy to treat NET patients.

Insight into angiogenic hallmarks

NETs are characterized for being highly vascularized tumors with the ability to produce large amounts of proangiogenic molecules. This is a feature of normal endocrine glands, which have a dense vascular network that facilitates hormone secretion and dumping to the bloodstream. In detail, some studies have shown that intratumoral vessel density is usually 10-fold higher in NETs than in carcinomas. Another trait of NET-composing cells is their capacity to synthesize and secrete high levels of the vascular endothelial growth factor (VEGF) (Capdevila *et al.* 2014). VEGF is the main mediator of tumor angiogenesis due to its effects as endothelial cell mitogen and vascular permeability-inducing agent.

Given that growing tumors require high amounts of oxygen and nutrients to allow the proliferation of tumor cells, tumor angiogenesis (the process by which blood vessels penetrate and grow inside the tumor) becomes critical for the survival of solid neoplasms, tumor invasion and metastatic dissemination. Additionally, there is a paracrine release of anti-apoptotic factors from activated endothelial cells of the newly formed vasculature that also contributes to tumor growth. Consequently, tumor cells tend to promote an event called angiogenic switch that occurs when the concentration of proangiogenic factors exceeds that of anti-angiogenic molecules (Hanahan & Folkman 1996). Therefore, when the shift in the balance of angiogenic inducers and inhibitors toward a proangiogenic outcome occurs, neovascularization inside the tumor is engaged.

However, the newly formed tumor blood vessels are structurally and functionally aberrant, presenting all the vascular components affected. In fact, tumor vessels show tortuosity, leakiness and lack of pericyte coverage, which leads to intratumoral hypoxia (Baluk *et al.* 2005, Jain 2005). Moreover, the endothelial cells composing the tumor vasculature are also abnormal, losing their polarity and allowing their detachment from the basement membrane which ends with their stacking upon each other and results in aberrant thickness of the

blood vessels walls (Baluk *et al.* 2005). Tumor endothelial cells also produce extensions into the lumen and form sprouts, with leading tip cells penetrating deep into tissue, thereby helping the invasion process. Additionally, these endothelial cells contain multiple fenestrations and other trans-endothelial channels, resulting in hemorrhage and increased interstitial fluid pressure.

Experimental data show that VEGF plays an important role in endocrine tumorigenesis and tumor progression. This has been well studied in a multistage pancreatic endocrine tumorigenesis model, the RIP1-Tag2 transgenic mouse, in which the angiogenic switch occurs in premalignant lesions and the neoangiogenic process persists during tumor progression. In the RIP1-Tag2 model, the transgenic mouse SV40 T-antigen (Tag) is expressed in endocrine beta cells under the control of the insulin promoter. The transgene converts normal insulin-producing beta cells of the pancreatic islets into islet cell carcinomas. Therefore, normal islets express the Tag oncogene, and RIP1-Tag2 mice are morphologically asymptomatic until 3–4 weeks of age. Hyperplastic islets start to arise stochastically (increasing to 50% of the islets by 10 weeks of age), showing beta-cell hyperproliferation, dysplasia characteristics and carcinoma *in situ*. In addition, switching of islet capillaries also forms angiogenic islets from these hyperplastic islets. This switch is characterized by endothelial proliferation, vascular dilation and microhemorrhaging. Tumor progression occurs by 12–13 weeks when the small encapsulated tumors (adenomas) progress into large adenomas and invasive carcinomas (Hanahan 1985). Tumor transition from endocrine hyperplasia to neoplasia is related to the acquisition of angiogenic properties (Hanahan & Folkman 1996), which can be blocked by the use of several anti-angiogenic agents (Bergers & Benjamin 2003). The RIP1-Tag2 model has been widely used to study the sensitivity of NETs to different anti-angiogenic drugs (Hanahan 1985). In fact, several anti-angiogenic treatments have been evaluated to prevent the angiogenic switch in premalignant lesions and restrict the rapid expansion of tumors.

Different therapeutic options exist for NETs patients depending on the localization of the primary tumors, the patterns of metastatic spread and the hormonal activity. The current standard procedure for localized disease is surgical resection, which still is the only curative strategy for some NETs. At the same time, treatments for patients with metastatic disease include control of tumor growth and alleviation of hormone-mediated symptoms (Kunz 2015).

However, conventional drugs are not selective for tumor cells and produce toxicity in normal tissues. Due to the limitations, vascular inhibitory targeting was proposed as a new approach to fight against tumor growth. Although the rationale of using angiogenesis inhibitors as anticancer drugs was received with skepticism among researchers when first presented by Dr Folkman in the 70s, (Folkman 1971), it soon startled an active research in the field, with the identification of several angiogenic inhibitors and subsequent clinical trials. This promising strategy leads to tumor cell death by the lack of oxygen and nutrient deprivation as a consequence of blood vessels. In this context, growth factors, their receptors and subsequent signaling cascades involved in vascular homeostasis are promising targets in angiogenesis inhibition.

Exploiting the current therapeutic repertoire

There are several factors such as tumor location, hormone secretion, invasiveness and metastasis that help to define the appropriate antitumor treatment. Since tumor survival

requires blood supply, different strategies can be used to reduce tumor vasculature. The vascular network can be inhibited at different levels, such as targeting endothelial cell proliferation, pericytes or extracellular matrix remodeling factors (Fig. 1). NETs exhibit overexpression of several growth factors and their receptors, such as VEGF, VEGFR and PDGFR (Terris *et al.* 1998, Fjallskog *et al.* 2003). Therefore, growth factors, their receptors and subsequent signaling cascades are promising targets in angiogenesis inhibition. In fact, many drugs have been developed to block growth factors and tyrosine kinase (TK) receptors.

Antivascular therapies have some advantages compared with conventional drugs. Chemotherapeutic drugs are not selective for tumor cells and produce toxicity in normal tissues with high proliferative rates such as the bone marrow, the gastrointestinal tract or the hair follicles. By contrast, anti-angiogenic therapies trigger cell death of tumors cells supplied by vessels. In the last years, numerous approaches have been made to evaluate the effects of anti-angiogenic agents and to understand their mechanism of action, most of which are still unknown.

Figure 1

Current and novel targets of anti-angiogenic strategies in tumor vessels. While semaphorin and VEGF/R inhibitors are described to act upon endothelial cells exclusively, FGFR inhibitors can act both on endothelial cells and tumor stroma. PDGF/R is a specific inhibitor of pericytes, while indirect anti-angiogenics (i.e. MTOR inhibitors) are specific inhibitors of non-vascular cells.

Importantly, many drugs have been developed and tested in well-differentiated NETs to block angiogenic growth factors and their receptors. Some of them, such as bevacizumab and sunitinib, have been already approved by the US Food and Drug Administration (FDA) for the treatment of some of these tumor types.

Current classical anti-angiogenic therapies: VEGF/R targeted therapies

Tumor cells can deregulate TK activity, thereby affecting many aspects of cellular function and conferring advantages to cancer cells, such as decreased apoptosis, increased proliferation, invasiveness and angiogenesis. TK inhibitors (TKIs) were postulated as a good approach for anticancer therapy due to the presence of a dual effect: they block both tumor cell proliferation and proangiogenic signaling pathways.

TKIs are small molecules with the ability to interact physically with the highly conserved kinase domains shared by different VEGFRs, as well as PDGF receptors, FGF receptors (FGFRs), EGF receptors (EGFR), Raf kinases and c-kit (a receptor of the pluripotent cell growth factor or stem cell factor), hence blocking the receptor activation (Gotink & Verheul 2010). TKIs can also be antibodies that bind to growth factors and prevent their binding to their receptors and their subsequent activation.

TK receptors are transmembrane proteins with an extracellular binding domain for ligands and an intracellular kinase domain. When the ligand binds the receptor, it is activated and stimulates an intracellular signaling cascade. The interaction between the inhibitor and the receptor directly inhibits tyrosine phosphorylation and the subsequent downstream proangiogenic signaling networks (Baka *et al.* 2006, Ivy *et al.* 2009).

Several TKIs have been developed for the treatment of many cancers. However, the efficacy of each inhibitor depends on the expression levels of the different growth factors or TK receptors they are blocking, and therefore different types of tumors may respond differently to each drug. The most important anti-VEGFR and anti-PDGF inhibitors are sorafenib, sunitinib and pazopanib (Gotink & Verheul 2010).

VEGF-targeted therapy Angiogenesis in PNETs and carcinoids is regulated by the influence of VEGF pathway (Kulke 2008, Raymond *et al.* 2011b). VEGF family is formed by six different members: VEGF-A, -B, -C, -D, -E and placental growth factor (PlGF), with different roles in

angiogenesis. VEGF can be produced by endothelial cells and promotes vascular homeostasis and development, or by tumor or stromal cells in a paracrine fashion, increasing vessel branching and tumor vessel abnormalities. VEGF levels are regulated by hypoxia, oncogene activation, downregulation of tumor suppressors, cytokines and growth factors levels. There are three different VEGFRs: -1, -2, -3, all of them expressed in endothelial cells. VEGFR1 was first identified and, although its function is still unknown, it may act as a decoy receptor, reducing the number of unbound, circulating VEGF available to bind VEGFR2. VEGFR1 might also have a role in endothelial cell recruitment and mitogenic signal induction. VEGFR2 is the low affinity receptor for VEGF, and it is known to be involved in angiogenesis and hematopoietic stimulation. The interaction between VEGF and VEGFR2 results in receptor dimerization, TK phosphorylation and activation of the signaling pathway involved in endothelial cell proliferation, migration and survival. It also contributes to the upregulation of molecules involved in extracellular matrix degradation.

VEGF pathway can be blocked by different strategies: inhibiting the VEGF ligand (e.g. bevacizumab), inhibiting the VEGFR (e.g. ramucirumab), soluble VEGFR/VEGFR hybrids (e.g. VEGF-Trap) and TKIs with selectivity for VEGFRs (e.g. sunitinib and sorafenib) (Fig. 1). The most important anti-VEGF drug is bevacizumab (Avastin; Genentech). Bevacizumab is a recombinant humanized monoclonal antibody against all isoforms of VEGF. It has been approved by the US Food and Drug Administration (FDA) for the treatment of several types of NET tumors but only in combination with other endocrine targeted therapies, such as octreotide (Yao *et al.* 2008b) or 2-methoxyestradiol (Kulke *et al.* 2010). For instance, a phase III clinical trial has been developed to test the effectivity of octreotide, a somatostatin analog, together with bevacizumab or INF α -2b in patients with metastasis or unresectable NETs (NCT00569127). In conclusion, the combination of bevacizumab and octreotide was associated with longer time to treatment failure (TTF) and more frequent radiologic response. Nevertheless, no significant differences in progression-free survival (PFS) were observed, suggesting that both bevacizumab and INF α -2b bare similar antitumor activity in patients with advanced NETs.

TK receptors, apart from being expressed in endothelial cells, are expressed in tumor and stromal cells. In addition, TKIs often target more than one type of receptors, so the inhibition of TK can affect various types of cells

(Krause & Van Etten 2005). Therefore, multitargeted TKIs, such as sorafenib and sunitinib, have demonstrated efficacy against various solid tumors in different clinical trials.

Sorafenib (Nexavar; Onyx Pharmaceuticals) is an oral synthetic compound with multiple targets, which inhibits angiogenesis and cell growth signaling. It has a dual effect since it can inhibit a component of the RAF/MEK/ERK signaling pathway, Raf-1, which controls proliferation and cell division. On the other hand, it can also inhibit VEGFR2–3 and PDGFR β signaling pathway, thus inhibiting angiogenesis (Llovet *et al.* 2008, Ivy *et al.* 2009, Raymond *et al.* 2011b). In a phase II clinical trial, sorafenib demonstrated a partial response of 10% in patients with progressive metastatic NETs (NCT00131911).

Another component is Sunitinib (Sutent; Pfizer), which is an orally administered small molecule that inhibits multiple targets such as VEGFR1–3, platelet-derived growth factor receptor α and β (PDGFR α – β), colony-stimulating factor-1 receptor (CSF-1R) and c-kit, thereby blocking angiogenesis and cell proliferation. It has been approved by the US Food and Drug Administration (FDA) and it has been tested in most of NETs in a phase III trial which resulted in an increase in patient progression-free survival (Raymond *et al.* 2011a, Delbaldo *et al.* 2012).

In the same line acts Pazopanib (Votrient). Pazopanib is an oral small molecule and multitargeted TKI that inhibits tumor angiogenesis by blocking VEGFR1, -2 and -3, PDGFR and stem cell factor receptor (c-Kit). Pazopanib produces dual effects on both tumor and endothelial cells, acting as an anti-angiogenic and anti-tumoral agent (Kumar *et al.* 2007, Hamberg *et al.* 2010, Ahn *et al.* 2013). Among others, a phase II clinical trial was recently carried out to assess the activity of pazopanib after failure of other systemic treatments in advanced NETs (NCT01280201). Pazopanib demonstrated clinical activity in patients with advanced NETs independently from previous treatments. Moreover, CTCs, soluble VEGFR-2 and VEGFR-3 gene polymorphisms were found to be potential biomarkers for selecting patients for pazopanib treatment (Grande *et al.* 2015). Another anti-VEGF drug is Vatalanib (Novartis), a TKI of all three VEGFR1–3 and several non-VEGF receptors, such as platelet-derived growth factor receptor (PDGFR).

The capacity to inhibit depends on the molecule and it is also proportional to the number of downstream affected targets. For example, Bevacizumab affects all the receptors influenced by VEGF, while sunitinib, pazopanib or vatalanib target the three VEGF receptors, as well as PDGF receptors and other non-VEGF receptors. This means that Bevacizumab has a modest inhibitory effect, contrary to the multitargeted inhibitors like sunitinib,

whose inhibitory capacity is profound across all cell, tissue and tumor types. These results led the investigators to conclude that blocking the classical VEGF pathway alone does not consistently alter neovascularization in tumors. Not only that, but more consistent results are obtained when multiple growth factor pathways are simultaneously affected.

Emerging targets in angiogenic therapy

Additionally to VEGF, several other growth factors and receptors are involved in tumor angiogenesis signaling processes of NETs including platelet-derived growth factor (PDGF), platelet-derived growth factor receptors α and β (PDGFR α and β), basic fibroblast growth factor (FGF), basic fibroblast growth factor receptor (FGFR), transforming growth factor α and β , epidermal growth factor (EGF), epidermal growth factor receptor (EGFR), guidance molecules (semaphorins), etc.

There is a need to explore different mechanisms regulating different components of vessels to understand their contribution to tumor progression and treatment response. Many of these pathways can be also inhibited by small molecules or by combination of different agents that target multiple proangiogenic pathways such as VEGF together with PDGF, FGF or EGF (Fig. 1).

PDGF-targeted therapies The PDGF family is composed of four polypeptide chains that assemble into five dimeric isoforms (PDGF-AA, PDGF-BB, PDGF-AB, PDGF-CC and PDGF-DD) that bind to two TK receptors (PDGFR α and PDGFR β) (Andrae *et al.* 2008). These molecules are implicated in pericyte coverage and can also regulate tumor vessel normalization. In fact, they have shown to have critical roles in cellular processes such as proliferation, survival and motility during tumor growth and invasion (Heldin & Westermark 1999). The proangiogenic PDGF and its receptor PDGFR work together in a paracrine manner: sprouting endothelial cells release PDGFB to chemoattract pericytes that express its receptor (Hellberg *et al.* 2010). Hence, PDGFR β acts as an attractant, stimulating cell migration, proliferation and cell fate. Consequently, the inhibition of PDGF signaling causes pericyte deficiency, leading to vessel leakage, tortuosity, microaneurysm formation and bleeding. Moreover, deficiencies in PDGFB or in pericytes form abnormal vessels.

Preclinical studies in patients have demonstrated that deficiencies in pericyte coverage disassemble the vessel wall and promote tumor invasion and metastasis

(Yonenaga *et al.* 2005). In addition, overexpression of PDGFD normalizes tumor vessels and increases drug delivery; while the inhibition of PDGFRB also improves drug delivery and the effectivity of chemotherapy (Hellberg *et al.* 2010). By monitoring tumorigenesis in the RIP1-Tag2-PDGFD knockout mouse model of pancreatic neuroendocrine tumors, it was shown that disruption of PDGF-DD signaling significantly delayed tumor growth. Therefore, aberrant platelet-derived growth factor receptor beta (PDGFR β) signaling in cancer has motivated the development of several antagonists currently used in the clinical setting such as imatinib (Novartis) (Fig. 1). Imatinib is an oral small molecule that affects the inhibition of ABL, c-kit and PDGFR signaling pathways. Due to its inhibitory potency, imatinib has significantly improved the treatment of cancers that crucially depend on the activation of these growth factor receptors. However, it has a low response ratio when compared to other anti-angiogenic agents, since it elicits its effects on PDGFR and lacks VEGFR inhibitory activity.

FGF-targeted therapies A second proangiogenic pathway involves the fibroblast growth factor (FGF) superfamily, formed by 23 different ligands and four TK receptors (FGFR1–4). The binding of an FGF to an FGFR induces receptor dimerization followed by receptor autophosphorylation and activation of downstream signaling pathways, activating the phosphorylation of several tyrosine residues in the COOH terminal tail, kinase domains and juxtamembrane region. This family elicits a dual action. On the one hand, it is involved in endothelial cell migration, proliferation and differentiation into a functional capillary vessel (Presta *et al.* 2005). For example, FGF1 and 2 directly promote endothelial cell proliferation, migration, vessel formation and maturation (Lieu *et al.* 2011). On the other hand, it also has an indirect effect concerning the regulation of the production of other proangiogenic molecules like VEGF, angiopoietin-2 (Ang-2) or interleukin-8 (IL-8) by tumor or stromal cells (Beenken & Mohammadi 2009). In addition to angiogenesis stimulation, FGF also regulates stromal fibroblasts and, consequently, produces an effect in tumor angiogenesis, since it is also an inducer of tumor cell proliferation via FGFR activation.

Blockade of FGF/FGFR signaling as a therapeutic approach to cancer is accomplished by either monoclonal antibodies that inhibit FGF binding by using small molecules that inhibit FGFR TK activity, or by allosteric modulators that adhere to the extracellular region of

FGFR domain (Fig. 1). There are some selective anti-FGFRs such as AZD4547 (AstraZeneca). AZD4547 is a highly active pan-FGFR selective inhibitor that suppresses FGFR signaling. Other selective inhibitors include BGJ398 (Novartis) that inhibits FGFR1–3, and LY287445, a selective pan-FGFR inhibitor. Anti-FGFR inhibitors are often weak, and for this reason, the combination of FGF inhibition with other drugs becomes necessary. In this context, since FGF signaling cooperates with VEGF pathway in tumor angiogenesis, the investigation of brivanib was prompted. Brivanib is a dual and selective FGF/VEGF inhibitor that was studied in the RIP1-Tag2 mouse model as a monotherapy. It showed blocking efficacy in the majority of samples analyzed and produced no signs of revascularization (Allen *et al.* 2011).

Other inhibitors that have been reported include Dovitinib, a potent TKI that also shows a potential anti-angiogenic action through the inhibition of FGFR, VEGFR and PDGFR; E3810, another potent dual inhibitor of VEGFRs and FGFRs; and Nintedanib, an oral compound with the capacity of blocking VEGFR, PDGFR and FGFR, showing highest activity against VEGFR1, 2, 3 and FGFR2.

It is evident that stromal cells play a key role in tumor angiogenesis and, consequently, in tumor progression, due to its function in matrix remodeling, tumor invasion and metastasis (Picard *et al.* 1986, Grey *et al.* 1989, Camps *et al.* 1990). Accordingly, the inhibition of these pathways could be an alternative to the classical anti-VEGF therapies.

EGF-targeted therapies Another proangiogenic pathway includes the epidermal growth factor (EGF)-like family of growth factors and their TK receptors (EGFRs): EGFR (ErbB1, HER1), ErbB2 (HER2, neu in rodents), ErbB3 (HER3) and ErbB4 (HER4). Upon ligand binding, receptor dimerization occurs, and the autophosphorylation of specific tyrosine residues takes place. These residues will then serve as binding sites for adaptor molecules and signal transducers. EGF family is involved in cell proliferation, differentiation, migration, adhesion, survival, inhibition of apoptosis and angiogenesis (Ritter & Arteaga 2003). EGF signaling pathway activation produces the release of proangiogenic factors such as VEGF, IL-8 and EGF from tumor and stromal cells. Therefore, EGF has a dual role: since they are expressed by tumor cells and endothelial cells, inhibitors of EGFR-activity can synergistically act as antitumor drugs too.

Some studies have shown that EGF expression is upregulated in NETs, where it is associated with

tumor proliferation and, therefore, correlates with poor prognosis (Shah *et al.* 2006). Consequently, anti-EGFR-based therapies have fantastic potential as anti-tumoral drugs. In addition to its effects on tumor cells, EGF is also expressed in vessels and its expression produces an increase in endothelial cell proliferation and survival. Moreover, it has been described that EGFR is expressed in tumor vessels of some types of cancer (Kedar *et al.* 2002, Holsinger *et al.* 2003, Sini *et al.* 2005, Thaker *et al.* 2005, Yokoi *et al.* 2005, Amin *et al.* 2006). Taken altogether, all these data suggest that apart from tumor cells, endothelial cells could be also target for anticancer therapy by EGFR-targeting drugs.

Some anti-ErbB therapies show inhibition of tumor angiogenesis in preclinical studies (Folkman 2007). For example, the administration of an EGFR inhibitor produces a decrease in EGFR on tumor vessels, which consequently generates a decrease in tumor angiogenesis due to an increase in endothelial cells apoptosis in some xenograft tumor models (Sini *et al.* 2005). Therefore, several strategies to inhibit EGFR have been developed and subsequent clinical studies have proven promising. There is a humanized anti-EGFR monoclonal antibody, cetuximab (IMC-C225), which targets the extracellular domain of the receptor, producing a downregulation of proangiogenic mediators accompanied by a reduction in vessel density and metastases (Ellis 2004, Huether *et al.* 2005). It has been reported that cetuximab suppresses neovascularization in some types of cancer such as epidermoid, lung, bladder, head and neck, and colon tumor xenografts (Petit *et al.* 1997, Perrotte *et al.* 1999, Huang *et al.* 2004, Morelli *et al.* 2006).

Another specific and reversible EGFR-TK inhibitor is Gefitinib (ZD1839), a synthetic anilinoquinazoline with a low molecular weight (Ranson & Wardell 2004). It blocks receptor autophosphorylation, induces cell cycle arrest and reduces cell proliferation in tumor cells that express EGFR. Some studies in neuroendocrine cell lines show a role for EGFR as a possible target and report efficacy of some EGFR inhibitors in NETs. Höpfner's group demonstrated *in vitro* that gefitinib produced a potent inhibition of the growth of NE gastrointestinal tumor cells by inducing cell cycle arrest and/or apoptosis, showing a promising role in inhibiting tumor growth (Höpfner *et al.* 2003). In addition to its direct effect on tumor cells, gefitinib has also an anti-angiogenic role through the inhibition of PI3K/Akt pathway, and it has been reported that it completely blocks neovascularization in some types of cancer (Iivanainen *et al.* 2009). This study also reveals that gefitinib regulates tumor angiogenesis by two

mechanisms: by reducing pericyte coverage of vessels, producing the instability of these new vessels; and by reducing the recruitment of BM-derived progenitor cells to the perivascular space of tumor vessels. In consequence, gefitinib could elicit its effect directly, blocking EGF and neovascularization, or indirectly, by inhibiting VEGF or interleukin-8 production.

Another molecule with similar properties to gefitinib is Erlotinib (Tarceva; Genentech, OSI Pharmaceutical, Roche). Erlotinib is an oral, potent and selective inhibitor of the EGFR (Moyer *et al.* 1997). This small molecule inhibits EGFR in a reversible manner, preventing the autophosphorylation of the receptor and, consequently, blocking its activity.

On the whole, these data suggest that tumors that express EGFR in both tumor and endothelial cells respond better to anti-EGFR therapy than those where only the tumor cells express the receptor (Baker *et al.* 2006). However, as monotherapy, some studies reveal that EGFR inhibitors have modest activity in solid tumors. The combination of EGF inhibitors either with conventional cytostatics or with other targeted agents could be the smartest strategy to block completely tumor angiogenesis (Klagsbrun *et al.* 2002, Huether *et al.* 2005, Moore 2005, Bourhis *et al.* 2006).

Semaphorin-targeted therapies Semaphorins are a large family of membrane-bound and secreted glycoproteins involved in guidance signaling pathways, in both attractive and repulsive activities, during the vascular and nervous system formation (Kolodkin *et al.* 1993, Luo *et al.* 1993). Moreover, they also have a role in cell adhesion and motility, angiogenesis, immune response and are key components of tumor progression (Tamagnone & Comoglio 2004, Zhou *et al.* 2008). Ligands within the semaphorin family are formed by a highly conserved extracellular domain, essential for semaphorin function, that mediates the binding to multimeric receptor complexes, mainly formed by plexins and neuropilins (NRPs) (Suchting *et al.* 2005). Semaphorins have been grouped into eight classes: classes 1 and 3 include invertebrate orthologues, whereas classes 3–7 are composed of vertebrate family genes, and class 5 is formed by viral semaphorins. Their receptors, plexins and neuropilins, are expressed in a variety of cell types such as endothelial cells, bone marrow-derived cells and cancer cells. In vertebrates, plexins are grouped in nine types of receptors, which are further divided into four subfamilies on the basis of overall similarity: plexin A (1–4), plexin B (1–3), plexin C1 and plexin D1. The extracellular

domain of plexins contains three PSI domains, whereas their cytoplasmic domain is highly conserved. Neuropilin receptors, grouped in NP1 and 2, are formed by a single-span transmembrane glycoprotein. They are associated with several intracellular signaling pathways involved in integrin function regulation, cytoskeletal dynamics, cell adhesion and migration.

As for tumor progression, semaphorins can act directly influencing the behavior of cancer cells, or indirectly, by modulating angiogenesis. Moreover, depending on their post-translational modifications, they can have a dual role in activating or inhibiting tumor progression and angiogenic response. For example, SEMA3A, 3B, 3E and 3F act as negative regulators of tumor angiogenesis (Klagsbrun *et al.* 2002, Schwarz *et al.* 2004), while SEMA 3CD and 5A promote tumor angiogenesis (Basile *et al.* 2004, Banu *et al.* 2006, Sadanandam *et al.* 2010b, Miyato *et al.* 2012).

Semaphorins as suppressors of tumor angiogenesis In developing vessels, the expression of Sema3A by endothelial cells produces the inhibition of angiogenesis, impairing endothelial cell migration and inducing cell apoptosis (Bates *et al.* 2003, Serini *et al.* 2003, Guttmann-Raviv *et al.* 2007). It has been described that Sema3A has a key role as an anti-angiogenic molecule; however, it has been shown that its expression is lost during tumor progression. In consequence, the overexpression of Sema3A could help to inhibit tumor growth and normalize the tumor vasculature. Some studies have shown that overexpression of Sema3A in different mouse models of cancer correlates with decreased tumor invasion (Herman & Meadows 2007, Casazza *et al.* 2011). More concretely, it has been shown that the re-expression of Sema3A in RIP-Tag2 tumors by adeno-associated virus improves tumor vascular function, reducing vascular density and increasing pericyte coverage, and prolongs the survival of treated animals (Maione *et al.* 2009, 2012). Thus, Sema3A re-expression normalized tumor vasculature, reducing the number of immature tumor blood vessels and re-established the normoxia in tumor tissue, thereby inhibiting tumor progression. Other studies show similar results in melanoma cells and in prostate cancer cells. In detail, it is described that the overexpression of Sema3A in these tumor types also suppresses tumor growth and tumor progression (Herman & Meadows 2007).

These data propose the overexpression of Sema3A as an attractive alternative approach for anticancer therapy in NETs. However, after some treatment cycles,

emerging resistance to these therapies has been described. In this context, the combination of Sema3A with other TKIs such as sunitinib could be an interesting strategy (Maione *et al.* 2012).

Another important Sema3 molecule with a potent anti-angiogenic activity is Sema3F. It has been shown to suppress tumor angiogenesis and metastasis in several cancer mouse models (Kessler *et al.* 2004). Similarly to Sema3A, Sema3F acts as a vascular normalizing agent, blocking peritumoral vessel sprouting, tumor cell adhesion and migration. Moreover, it has also been described as a metastasis suppressor in different animal models (Bielenberg *et al.* 2006).

Sema3E is also involved in the regulation of tumor angiogenesis and cancer progression. In fact, its re-expression in a xenograft melanoma model strongly reduces metastasis formation (Roodink *et al.* 2008). Finally, Sema3B in addition to its direct antitumor effect can also acts as an angiogenic inhibitor (Varshavsky *et al.* 2008).

These findings further corroborate that the aberrant vasculature in tumors is a direct consequence of an imbalance between the pro- and anti-angiogenic factors. Therefore, the restoration of this equilibrium in tumors by re-expression of Sema3A, E and F, is crucial for tumor vasculature normalization and, consequently, tumor growth control.

Semaphorins engaging tumor angiogenesis Apart from their beneficial role as tumor angiogenesis inhibitors, it has been shown that some semaphorins also contribute to trigger tumorigenesis and metastasis (Basile *et al.* 2004, 2006, Wang *et al.* 2015).

The most studied proangiogenic Sema is Sema4D, which promotes tumor angiogenesis by inducing endothelial cell proliferation, adhesion and migration, as well as Sema3C, whose expression in some types of cancer has been correlated with tumor progression (Galani *et al.* 2002, Banu *et al.* 2006, Herman & Meadows 2007). Sema3C promotes tumor migration and is highly expressed in metastatic tumor cells. Semaphorin 3C also promotes endothelial cell proliferation, migration and tube formation.

Sema4D exerts its proangiogenic activity through binding to its PlexinB1 receptor in endothelial cells (Basile *et al.* 2004). Regarding the signaling mechanisms, two alternative pathways have been proposed. While one involves RhoA activation, engagement of integrins and activation of TKs (Basile *et al.* 2005, 2007a), the other

proposes Met activation upon Sema4D-PlexinB1 as the triggerer of TK phosphorylation cascade (Conrotto *et al.* 2005). In both cases, Sema4D-mediated activation leads to cytoskeletal reorganization in endothelial cells and an increase in their migratory properties.

Considering its role in angiogenesis, the potential association of Sema4D with tumor angiogenesis was studied and it was found to be highly expressed in different type of tumors such as breast, head, neck and oral (Basile *et al.* 2006, Ch'ng *et al.* 2007). Moreover, several clinical studies have showed that a higher Sema4D expression is correlated with higher necrosis ratio, mitotic ratios and poorer prognosis (Ch'ng & Kumanogoh 2010).

At the molecular level, induction of angiogenesis starts with the cleavage of the extracellular portion of Sema4D by matrix metalloproteinase type 1 (MT1-MMP, also known as MMP14) and binds to PlexinB1 or to other lower affinity receptors such as PlexinB2, allowing it to act on local and distant tumor niches (Basile *et al.* 2007b, Fazzari *et al.* 2007). On the other hand, Sema4D can regulate tumor-associated macrophages (TAMs) and other monocytic cells of the tumor stroma. In this context, Sema4D showed an inhibitory effect on cell migration (Delaire *et al.* 2001). These results could be interpreted as a mechanism of tumor cells for the retention of immune cells in the tumor stroma, further potentiating inflammation and cytokine production.

Regarding the way forward to the clinic for semaphorin targeting, an antibody against anti-Sema4D, MAb67, has been developed (Vaccinex Inc, Rochester, NY, USA). In preclinical studies, anti-Sema4D/MAb67 has shown to inhibit tumor growth by promoting immune infiltration and reducing immunosuppression (Evans *et al.* 2015). These results, together with PK/KD, safety and immunolocalization studies in rat and cynomolgus monkeys, supported the initiation of a phase I clinical study to assess the safety and tolerability of VX15/2503 (an MAb67-derived humanized antibody) in patients with advanced solid tumors (NCT01313065). Another proangiogenic molecule is Sema5A, a transmembrane semaphorin which binds to PlexinB3, increasing angiogenesis, tumor growth, invasion and metastasis (Pan *et al.* 2010, Sadanandam *et al.* 2010b), and is a putative metastatic marker for pancreatic cancer (Sadanandam *et al.* 2007). It has been demonstrated that Sema5A, as a proangiogenic molecule, can regulate several processes during angiogenesis, such as endothelial cell proliferation, survival and migration and sprouting of blood vessels (Sadanandam *et al.* 2010a). In fact, it has been shown that

secreted Sema5A enhances invasiveness and metastasis of tumor cells via ERK phosphorylation, while proliferation of endothelial cells is promoted through upregulation of angiogenic factors (Sadanandam *et al.* 2012).

To sum up, Semaphorin family members are essential regulators of the tumor microenvironment, since their expression is often altered in tumor samples compared to normal tissues. Importantly, the expression of certain inhibitory semaphorins is decreased in some aggressive tumor cells, supporting the idea that they work as tumor suppressing genes. In contrast, proangiogenic and tumorigenic semaphorins are upregulated during malignant processes, which may indicate that their inhibition could be a potential therapeutic approach (Fig. 1). On account of the data resumed above, currently, semaphorins are highlighted as alternative anti-angiogenic targets that if adequately inhibited or activated could have the potential to impair tumor growth (Pircher *et al.* 2014).

Targeted therapies with indirect anti-angiogenic effects

There are some molecules that, although apparently not directly involved in any angiogenic pathway, may exert their antitumor activity through indirect anti-angiogenic effects. This is the case of mTOR inhibitors and somatostatin analogs (Fig. 1). mTOR is an intracellular serine–threonine kinase that regulates cell signaling processes controlling apoptosis, proliferation, cell growth and metabolism. In addition to their multiple roles, mTOR inhibitors also demonstrate anti-angiogenic properties. Upon the activation of the signaling pathway, PI3K triggers the generation of phosphatidylinositol 3,4,5-triphosphate (PIP3), which provokes the subsequent activation of AKT, a serine/threonine kinase that activates multiple cellular target proteins. It has been shown that mTOR activation may participate in pancreatic NET progression (Missiaglia *et al.* 2009). Therefore, the inhibition of mTOR pathway is a promising strategy for drug therapy.

The natural antibiotic rapamycin (sirolimus) is one of such inhibitors of mTOR and acts as a tumor suppressor. There are two analogs of rapamycin: temsirolimus (Wyeth Pharmaceuticals), a soluble ester analog involved in cell cycle inhibition whose use was abandoned after the first trials (Duran *et al.* 2006, O'Donnell & Ratain 2007); and everolimus (Novartis), with a high oral bioavailability and potential anti-angiogenic properties. Everolimus has been tested successfully for its anti-neoplastic potency, and it has been approved for its use in patients with advanced pancreatic NETs, since it significantly improved

progression-free survival among patients with advanced pancreatic NETs (Yao *et al.* 2013, 2015). Everolimus is involved in the inhibition of VEGF synthesis and secretion, together with the production of HIF α by the tumor cells. It specifically affects endothelial cell survival by inducing endothelial cell apoptosis and inhibiting endothelial progenitor cell proliferation and differentiation. In conclusion, the inhibition of mTOR pathway indirectly affects the new vessel formation process, resulting in dual, direct and indirect, tumor growth impairment.

Another example of non-targeted therapies with possible anti-angiogenic properties is somatostatin analogs (SSAs). Somatostatin is a peptide hormone with the ability to prevent hormone release and cell growth after binding to its receptors (sst₁–sst₅). Due to the short half-life of native somatostatin and the rebound hormone hypersecretion, clinically useful analogs have been developed. The best known SSAs approved by the FDA for NET treatment are octreotide (Sandostatin; Novartis) and lanreotide (Somatuline; Ipsen). Besides their antiproliferative effect on tumor cells, SSAs demonstrate strong anti-angiogenic properties *in vitro* by inhibiting endothelial cell proliferation and the synthesis and secretion of several angiogenic factors such as VEGF either in stromal or tumor cells (Danesi & Del Tacca 1996, Woltering *et al.* 1997). Nevertheless, the translation of the *in vitro* anti-angiogenic properties of somatostatin and SSAs to the *in vivo* setting and its potential exploitation in human disease remains controversial, and little evidence exists (Walter *et al.* 2011).

Overcoming the hurdles and shaping the future of NET treatment

Some of the main characteristics of NETs are their high vascularization and their capacity to synthesize and secrete high levels of VEGF. Due to the limitations of chemotherapeutics, the use of anti-angiogenic molecules to try to inhibit neovessel formation to fight against tumor growth is currently explored. In short, tumor angiogenesis inhibition will engage tumor cell death via lack of oxygen and nutrient deprivation.

Angiogenesis is a complex process in which several biological mechanisms are involved: endothelial cell proliferation, vessel guidance, vessel maturation, stabilization and quiescence. During all these steps, there is an interplay between different molecular families, including growth factors, migratory cues and other signaling molecules, which is crucial to understand the

process of tumor angiogenesis as a promising target to fight against cancer. It has been shown that the blockade of endothelial cells, tumor stroma cells (pericytes, fibroblast and immune cells) or extracellular matrix remodeling molecules produces tumor blood vessel reduction. Hence, due to the high number of molecules and different cell types involved in vessel formation, angiogenic processes can be inhibited at different levels (Fig. 1). Nevertheless, the canonical VEGF pathway is the most studied one and, for this reason, many inhibitors have been developed to block the action of VEGF or its receptors, VEGFRs.

Even though many angiogenesis inhibitors have been described, only some of them have been approved and used in clinics. Furthermore, the clinical benefit of anti-angiogenic drugs remains limited due to the acquisition of drug resistance by stromal cells, and they need to be improved to guarantee patient's long term response. An example of the therapy failure was observed in a preclinical study using the RIP1-Tag2 mouse model of pancreatic islet carcinogenesis. The initial efficacy of anti-angiogenic therapy *in vivo* was then followed by the acquisition of resistance as demonstrated by tumor revascularization, regrowth and invasiveness (Casanovas *et al.* 2005). Efforts for a better understanding of the mechanism underlying resistance to therapy aided to describe intrinsic (primary) and adaptive (secondary) resistance as the main modes of non-responsiveness to antitumor therapy. While intrinsic resistance refers to naturally non-responder tumors to anti-angiogenic therapies such as sunitinib and bevacizumab (Edelman & Mao 2013), acquired or adaptive resistance stems from tumor adaptations to therapy. The latter includes the creation of alternative mechanisms that lead to additional proangiogenic signaling pathways activation (Jimenez-Valerio & Casanovas 2016). Current notion regarding anti-angiogenic resistance has led to the conclusion that the blocking of the classical VEGF pathway alone does not consistently alter neovascularization in tumors, and more efficient results are obtained when multiple growth factor pathways are simultaneously affected.

There is a need to identify and characterize additional molecular regulators of angiogenesis in order to develop new drugs. It has been described that, during tumor evolution, different angiogenic molecules are expressed at different levels depending on the tumor progression status. For instance, during the early stages of breast cancer, tumors require only VEGF for angiogenesis, whereas at later stages, other factors such as FGF and PDGF are needed. Therefore, a late-stage breast tumor

cell may not respond to classical anti-VEGF treatment because alternative angiogenic factors are involved in the angiogenesis process. In this context, combinatorial strategies are also required to disrupt several receptor and non-receptor kinases in association with classic clinical drugs such as anti-VEGF. In the future, new drugs targeting PDGF, FGF, EGF or semaphorin pathways will help to improve the therapeutic effect against cancer cells, vascular and other stromal cells in NETs (Fig. 1). In conclusion, in order to fight against tumor progression, normalize tumor vasculature and avoid drug resistance and tumor regrowth, there is an increasing urge to combine several vessel formation-targeted therapies. The strategy is based on the combination of therapies that target multiple components of the tumor stroma in order to inhibit tumor growth from different stages of vessel formation. Nevertheless, clinical trials combining drugs targeting two crucial pathways such as VEGF and mTOR have demonstrated undesirable side effects in terms of efficacy due to an increased toxicity. For example, a phase II study of the combination of sorafenib and bevacizumab for patients with advanced NETs resulted in a global PFS rate of 90.9% (Castellano *et al.* 2013). Unfortunately, unfavorable safety results were showed in comparison to these same drugs in monotherapy, arguing that a sequential approach should be used instead. On the contrary, new preclinical and clinical data are giving evidence of antitumor efficacy of dual inhibition in the presence of moderate toxicity in other types of tumors such as metastatic renal cell carcinoma (Motzer *et al.* 2016). In the future, further research in the field of angiogenesis will allow the development of new combinatorial strategies where pharmacological toxicity is limited. Multi-targeting will, undoubtedly, lead to reduced resistance and improved therapeutic outcome in patients who are receiving anti-angiogenic therapy.

Declaration of interest

Oriol Casanovas declares that he has been economically compensated for his assistance to advisory boards and conferences from Novartis, Pfizer, Ipsen and Teva. Apart from this, there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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