Combination Therapies Targeting PDGF and VEGF Signaling Pathways in Solid Tumors

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Abstract

Vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) are independently involved in several cancer-associated mechanisms including autocrine stimulation of cancer cells, stimulation of tumor angiogenesis and regulation of interstitial fluid pressure (IFP). The scope of this thesis was to investigate the combinatory effect of anti-VEGF and anti-PDGF treatment on tumor angiogenesis and tumor IFP.

Angiogenesis is a process of formation of blood vessels. Based on the tumors dependency on the blood vessels to supply them with oxygen and nutrients, several anti-angiogenic therapies have been tried and shown to have beneficial anti-tumor effects. More recently, anti-angiogenic treatment appeared to transiently “normalize” disorganized tumor vasculature and therefore to improve the uptake of cytotoxic agents.

In the first study, treatment was performed on two tumor models that differ only with regard to the degree of maturation of the vasculature, reflected by different number of pericytes that are the target for anti-PDGF treatment in these tumors. The aim was to study the role of pericyte coverage in protecting endothelial cells from anti-VEGF therapies. In the pericyte-rich tumor model the combination treatment gave a more efficient anti-angiogenic effect. Interestingly, it was only a subset of pericytes that was sensitive for the treatment.

In the second paper, the effect of anti-VEGF and anti-PDGF treatment on tumor IFP was measured. IFP is elevated in most solid tumors, which is linked to poor prognosis and higher recurrence rate. Additionally, this serves as a problem in ant-cancer therapies since it makes the uptake of cytotoxic agents inefficient. PDGF is known to actively regulate the IFP by regulating the contractile activity of fibroblasts, while VEGF regulates IFP primarily by affecting vessel leakiness. In the current study, combination of anti-VEGF and anti-PDGF therapies was shown to have an additive effect. However, the timing of administration of inhibitors appeared to be crucial. It was only short, but not long term combination treatment that further reduced IFP as compared to monotherapies. Surprisingly, the additive effect on IFP did not translate into an increased efficiency of chemotherapy when comparing combination treatment with monotherapies.

The last paper is a follow up of the first study, where it was shown that combination of anti-VEGF and anti-PDGF treatment affect the tumor vasculature. Here we investigated if the anti-angiogenic effect improves treatment efficiency of a cytotoxic agent. There was a significant effect of the combination of anti-VEGF and anti-PDGF on Taxol treatment efficiency in this Taxol resistant tumor model. However, the mechanism for the treatment effect and the relative contribution of the targeted vasculature in the outcome of the therapy remains to be determined, since tumor cells were also sensitized for Taxol in vitro.

In summary, we have shown that targeting of PDGF and VEGF signaling pathways simultaneously affect both vasculature and IFP to a higher extent than monotherapies.

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urn:nbn:se:uu:diva-119827 (http://urn.kb.se/resolve?urn=nbn:se:uu:diva-119827)
To Piotrek, Staś, the Little One

and

to my whole wonderful Family
She’s been in this world for over a year, 
and in this world not everything’s been examined 
and taken in hand.

The subject of today’s investigation 
is things that don’t move themselves.

They need to be helped along, 
shoved, shifted, 
taken from their place and relocated.

They don’t all want to go, e.g., the bookshelf, 
the cupboard, the unyielding walls, the table.

But the tablecloth on the stubborn table 
- when well-seized by its hems –
manifests a willingness to travel.

And the glasses, plates, 
creamer, spoons, bowl, 
are fairly shaking with desire.

It’s fascinating, 
what form of motion will they take, 
once they’re trembling on the brink: 
will they roam across the ceiling? 
fly around the lamp? 
hop onto the windowsill and from there to a tree?

Mr. Newton still has no say in this. 
Let him look down from the heavens and wave his hands.

This experiment must be completed. 
And it will.

Wisława Szymborska
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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Abbreviations

ASMA  Alpha-smooth muscle actin
CAM  Chick chorioallantoic membrane
CAFs  Cancer-associated fibroblasts
CML  Chronic myelogenous leukemia
CNS  Central nervous system
CXCR4  Chemokine receptor-4
DDR  Discoidin domain receptor
DFSP  Dermatofibrosarcoma protuberans
EMT  Epithelial-to mesenchymal transition
EPCs  Endothelial progenitor cells
ERK  Extracellular-signal-regulated kinase
FGF  Fibroblast growth factor
GIST  Gastrointestinal stromal tumor
HGF  Hepatocyte growth factor
HIF  Hypoxia-inducible factor
IFP  Interstitial fluid pressure
IL-8  Interleukin 8
MEK  ERK kinase
NRP1  Neuropilin-1
PDGF  Platelet-derived growth factor
PDGFR  Platelet-derived growth factor receptor
PI3K  Phosphoinositide 3-kinases
PLCγ  Phospholipase Cγ
PIGF  Placental growth factor
RTKs  Receptor tyrosine kinases
SDF-1  Stromal-derived growth factor-1
SH-2  Src homology-2
TAF  Tumor angiogenesis factor
TGFβ  Transforming growth factorβ
VEGF  Vascular endothelial growth factor
VEGFR  Vascular endothelial growth factor receptor
VPF  Vascular permeability factor
1. Introduction

The main motivation of cancer research is to understand the disease well enough to be able to alter its course since it is still a major cause of human death worldwide. So far, surgery, radiotherapy and chemotherapy have been the main strategies to cure cancer. Unfortunately, despite many developments in these treatments, many types of cancer are still incurable. The success of surgery depends on the maturity of the adjacent neovascular structure and on the location of tumor. The limitation of radiotherapies and chemotherapies is that they target also nonmalignant cells leading to severe side effects like mucositis, hair loss and bone marrow suppression. Chemotherapies are based on the fact that cancer cells proliferate whereas most normal cells do not. Since tumor is composed of heterogeneous population of cells, those that have low proliferating index may become chemoresistant.

Cancer involves changes in the genome and is characterized by alterations in cell physiology including: self-sufficiency for growth signals, insensitivity to inhibitory signals, evasion of apoptosis, indefinite replicative potential, sustained angiogenesis, tissue invasion and metastasis (Hanahan et al. 2000). Malignant cells exhibit perturbations of signaling pathways that maintain normal functions of multicellular organisms (Blume-Jensen et al. 2001). The proper treatment of cancer patients requires detailed understanding of the mechanisms of such perturbations. This knowledge may lead to therapies targeting specifically cancer cells, leaving unaffected normal cells from harmful effects (Dancey et al. 2003).

Receptor tyrosine kinases (RTKs) constitute of a family of growth factor receptors that transmit the extracellular signal to the cells, and control crucial biological functions such as cell proliferation, differentiation and apoptosis (Schlessinger 2000). More than half of RTKs have been found to be mutated or activated in several malignancies (Blume-Jensen et al. 2001). They also participate in modulating tumor environment by promoting tumor angiogenesis and regulating the interstitial fluid pressure (IFP) that accelerates a malignant phenotype and leads to metastasis. That is why there has been an increasing interest in RTKs as drug targets in cancer therapy. Two very important RTKs for cancer progression are the platelet-derived growth factor receptor (PDGFR) and vascular endothelial growth factor receptor (VEGFR). Drugs affecting either one or both of them are already in clinical use, with encouraging treatment effects for certain tumor types.
2. Role of VEGF and PDGF signaling pathways

2.1. Signal transduction through VEGF receptors

The VEGFR family belongs to the fifth class of RTKs and consists of six or seven extracellular immunoglobulin-like domains and intracellular domain with kinase activity. VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1) and VEGFR-3 (Flt-4) bind five different mammalian ligands: vascular endothelial growth factor-A (VEGF-A), VEGF-B, VEGF-C, VEGF-D and placental growth factor (PIGF) as presented in Figure 1 (Olsson et al. 2006). In addition, VEGF like factors have been described for parapoxvirus (VEGF-E) and have been found in the snake venom (VEGF-F) (Olsson et al. 2006). VEGF-A exists in four different splice variants comprising 121, 165, 189 and 206 amino acid residues (mouse variants are one amino acid residue shorter), with different biological activities. VEGF121 lacks the region binding to heparan sulfate in the extracellular matrix and is freely diffusible, while other isoforms bind heparan sulfate to different extents. In addition, there are several accessory receptors that modulate signal transduction, like neuropilins that increase the binding affinity of the VEGF ligands (Olsson et al. 2006).

*Figure 1. Binding specificity of mammalian VEGF ligands to VEGF receptors.*
Like in other RTKs, signaling by VEGFRs is initiated by ligand binding to the extracellular domain of the receptors. This leads to dimerization of the receptor and phosphorylation of specific tyrosine residues localized in the activation loop, the kinase insert and C-terminal tail of the receptor (Figure 2). As a consequence, a variety of molecules are recruited allowing for the assembly of a large molecule complexes that activate several signaling pathways, including phosphoinositide 3-kinases/Akt (PI3K/Akt), phospholipase Cγ (PLCγ) and Ras/Raf/Mitogen-activated protein kinase/ERK kinase (MEK)/extracellular-signal-regulated kinase (ERK) (Ras/Raf/MEK/ERK) leading to proliferation, migration and survival of endothelial cells. The interactions with the receptor are mediated predominantly through Src homology-2 (SH-2) domains, which bind to phosphorylated tyrosine residues (Olsson et al. 2006).

Figure 2. Structure of the intracellular domain of VEGFR-2 with major phosphorylation sites and mechanism of the tyrosine phosphorylation.

The termination of signaling through VEGFR is controlled by dephosphorylation by phosphatases followed by degradation of the receptor in proteasomes and lysosomes (Olsson et al. 2006).

2.2. Physiological role of VEGF signaling

2.2.1. Angiogenesis, vasculogenesis, lymphangiogenesis, hematopoiesis

The development of the vascular system is one of the first events during organogenesis. It takes place in successive steps named vasculogenesis and angiogenesis. Vasculogenesis is the process of de novo specification of he-
mangioblasts form the mesoderm into the primary vascular plexus (Figure 3). This network undergoes a process called angiogenesis which involves sprouting, bridging and branching by intussusception of pre-existing vessels, i.e. the division of one vessel into two by the formation of the new vessel wall in the lumen of the original one (Burri et al. 2004). In consequence primary vascular plexus remodels into a vascular tree composed of arteries and veins. There are several specialized endothelial cells that regulate the formation of a functional branch including: endothelial “tip”, “stalk” and “phalanx” cells (De Smet et al. 2009). Lymphatic vessels are mainly formed from veins during a process called lymphangiogenesis, but also from lymphangioblasts (Wilting et al. 2006). Hematopoiesis is the formation of blood cellular components form the precursor hemangioblast. Vasculogenesis, angiogenesis, lymphangiogenesis and hematopoiesis are regulated by VEGF signaling pathways (Figure 3).

**Figure 3.** Schematic illustration of steps in vascular development, lymphangiogenesis and hematopoiesis.

The importance of VEGFs and their receptors in development of the vasculature was confirmed by knock-out studies in mice. The knock-out of just one VEGF-A allele leads to embryonic lethality at day 11-12, due to lack of functional vasculature and the absence of blood islands (Carmeliet et al. 1996; Ferrara et al. 1996). A similar phenotype, with absence of functional endothelial cells and some subtypes of hematopoietic cells was observed in VEGFR-2 -/- mice leading to lethality between days 8.5 to 9.5 (Shalaby et al. 1995). In VEGFR-1 deficient mice endothelial cell differentiation was normal, but the vasculature was disorganized leading to embryonal death on days 8.5-9 (Fong et al. 1995; Fong et al. 1999a). VEGFR-1 is considered as negative regulator for VEGFR-2, functioning as a “trap” for VEGF-A. Deletion of the tyrosine kinase domain of VEGFR-1 does not affect the vascular development per se (Hiratsuka et al. 1998). VEGFR-3 has a crucial role in
the establishment of lymphatic vessels (Shibuya et al. 2006). However, the mutation of VEGFR-3 leads to embryonic lethality before the formation of lymphatics due to cardiovascular failure (Dumont et al. 1998).

The expression of VEGFR-2 is already observed at very early stages of development (Yamaguchi et al. 1993). The lateral and posterior mesodermal cells migrate toward the yolk sac. During the migration, the precursors cluster into so called hemangioblastic aggregates that will subsequently differentiate into endothelial cells and hematopoietic cells (Figure 3) (Eichmann et al. 1997). It was observed that individual VEGFR-2 positive cell could differentiate either into endothelial cells or into hematopoietic cells, but not both (Eichmann et al. 1997). However, in other studies, the activity of a single cell to differentiate into both lineages was observed (Choi et al. 1998). Thus, it remains to be established whether endothelial cells and hematopoietic cells share a common precursor. Moreover, it was also observed that in the presence of PDGF, VEGFR-2 positive cells could give rise to smooth muscle cells (Yamashita et al. 2000) indicating that rather than being strictly programmed to only endothelial cells or hematopoietic cells, these cells may be pluri- or multi-potent progenitors. More recently, in a study on zebrafish embryos, it was shown that not all endothelial cells and hematopoietic cells are derived from the same common progenitor (Vogeli et al. 2006).

The mechanism of vessel navigation remains still incompletely understood. It is, however, known that endothelial tip cells are located in the front of a growing vessel. In the response to chemo-attractant they extend filopodia to form capillary sprouts (Gerhardt et al. 2003). Vascular guidance is a general phenomenon in vascular development as well as during pathological angiogenesis. The role of VEGF-induced tip cell guidance is based on studies with mouse mutants that selectively expressed different VEGF splice variants (Ruhrberg et al. 2002). VEGF164 which can bind to extracellular matrix and also is freely diffusible can provide proper patterning (Stalmans et al. 2002). In contrast, mice expressing either VEGF120 that lacks heparin-binding motif and is only freely diffusible form, or VEGF188 that can bind to extracellular matrix lead to defects in the vessel navigation. Mice with VEGF120 had vessels that increased in size rather than branching. In contrast, VEGF188 showed the opposite phenotype with hyperbranched and thin vessels (Ruhrberg et al. 2002). Therefore, the proportion of different VEGF isoforms as gradient in the matrix is crucial for the balance between growing and branching of blood vessels. Additionally, application of soluble VEGF to early chick embryos lead to hyperfusion, while administration of soluble VEGF-1 cause inhibition of tip cell protrusions (Drake et al. 2006). Stalk cells, another type of endothelial cells, follow the leading tip cells. They proliferate, elongate the stalk, form a lumen and connect to the circulation. Notch signaling is very important in these cells since it impairs filopodia extension and inhibit vessel branching by lowering the expression of VEGFR-2, VEGFR-3, neuropilin-1 (NRP1) and chemokine receptor-4 (CXCR4) (De Smet et al. 2009). Phalanx cells, the most quiescent, are cov-
pered by pericytes and stick to each other by tight junctions. They are involved in optimizing blood flow, tissue perfusion and oxygenation. VE-cadherin is involved in the switch of the response to VEGF by endothelial cells from proliferation and migration to survival and quiescence (De Smet et al. 2009).

2.2.2. Organogenesis
Since organs contain blood vessels, VEGF plays a crucial role in organ formation during development. A role of VEGFR-2 in liver development was found through studies on VEGFR-2 knock-out mice. In these animals there was no migration and proliferation of hepatoblast that form the liver bud (Matsumoto et al. 2001). VEGF also promote proliferation of hepatocytes providing essential supportive role to a damaged adult liver (LeCouter et al. 2003). Mutual signaling between endothelial cells and endocrine cells occurs in order to establish well-function pancreas (Lammert et al. 2001; Lammert et al. 2003). Signaling between endothelial cells and podocytes is essential for proper development and maintenance of the filtration function of the kidney glomerulus (Mattot et al. 2002; Eremina et al. 2003). Mice lacking PlGF have no physiological defects, but have impaired angiogenesis, plasma extravasation and collateral growth during ischemia, inflammation, wound healing and cancer (Carmeliet et al. 2001).

An additional protective role of VEGF signaling has also been observed in non-endothelial cells like neurons. VEGF protects motor neurons under stress conditions in vitro (Oosthuyse et al. 2001). Lack of VEGF-A in a population of neural cells, obtained by a Nestin-Cre mouse line, that specifically lower VEGF-A levels in the central nervous system (CNS), causes abnormalities in retina and cortex development. However, in the last case, the effect was considered to be a secondary effect of inappropriate vascular invasion (Haigh et al. 2003). This suggests that VEGF might have a dual role in the nervous system; a direct neurotrophic role and a pro-angiogenic role leading to proper perfusion and delivery of various neurotrophic factors. VEGF signaling has also a crucial role in bone development, promoting vascularization during endochondral bone formation (Maes et al. 2004) and regulating prosurvival activity of chondrocytes (Zelzer et al. 2004) and osteocytes (Nakagawa et al. 2000). Other non-endothelial targets for VEGF include myoblasts (Arsic et al. 2004) and pneumocytes (Compernolle et al. 2002).

2.3. Signal transduction through PDGF receptors
PDGFs are a family of growth factors (PDGF-AA, -AB, -BB, -CC and -DD) (Fredriksson et al. 2004) that stimulate cell growth, survival and motility through activation of PDGFRα and PDGFRβ which belong to the third class
of the RTKs (Figure 4) (Heldin et al. 1998). A stretch of amino acids residues in the C-terminus of PDGF-AA and PDGF-BB, called the retention motif, is required to restrict the diffusion of the ligand. Deletion of this motif in PDGF-BB causes the detachment of pericytes from the vascular bed (Lindblom et al. 2003). PDGF-CC and PDGF-DD contain an additional domain called CUB domain. They are secreted as latent factors and in order to be active they need to undergo a proteolytical cleavage (Fredriksson et al. 2004).

Similar to VEGF, PDGF binding cause receptor dimerization and autophophorylation, allowing binding and activation of several cytoplasmic signal transduction molecules containing SH-2 domains (Heldin et al. 1998). Thereby, a number of different signaling molecules, including adaptor proteins like Shc and Grb2, enzymes such as Src, PI3K, PLCγ and the tyrosine phosphatase like SHP-2 are activated, bind to the receptors and thereby initiate signaling pathways leading to cell differentiation, proliferation, apoptosis and migration (Heldin et al. 1998). PDGFR signaling is terminated by receptor internalization followed by receptor dephosphorylation through tyrosine phosphatases (Markova et al. 2003; Persson et al. 2004) and degradation in proteosomes and lysosomes (Haglund et al. 2003).

![Figure 4. Binding specificity of PDGF ligands to PDGF receptors.](image-url)
2.4. Physiological role of PDGF signaling pathway

The discovery of PDGF isoforms in the 1970s was followed by numerous experiments showing that the PDGF family act as mitogens for many cell types of mesenchymal or neuro-ectodermal origin (Heldin et al. 1999). The importance of PDGF signaling was confirmed by in vivo studies using knock-out, knock-in and transgenic mutant mice (reviewed in (Betsholtz 2004)). PDGF-BB and PDGFRβ knock-out mice die from bleedings (Leveen et al. 1994; Soriano 1994), caused by the shortage of vascular mural cells and pericytes (Lindahl et al. 1997; Hellstrom et al. 1999). Pericytes are smooth muscle cells that are found in close contact with endothelial cells within the basement membrane. They not only provide mechanical support for the vessel wall, but they also release pro-survival factors for the endothelium such as angiopoietin-1 and transforming growth factor β (TGFβ) (Armulik et al. 2005). Endothelium is the most important source of PDGF-BB for pericyte recruitment (Enge et al. 2002) and the strongest production of VEGF occurs in the endothelial tip cell (Gerhardt et al. 2003). The amount of pericytes is highest in retina. However, one cannot study the pericyte recruitment in retina in neither PDGF-B nor PDGFRβ knock-out mice due to embryonic lethality, since the retina develops postnatally. Some other available models like EC-PDGF-BB KOs (endothelium-restricted deletion of PDGF-B gene), PDGF-B ret/ret (mice with deleted retention motif on PDGF-BB) and PDGFRαβ/- mutant mice (PDGFR-targeted mice with intracellular domain of the PDGFRα replaced by PDGFRβ and vice versa) display reduced pericyte coverage and retinopathy (Klinghoffer et al. 2001; Enge et al. 2002; Lindblom et al. 2003). PDGF-BB or PDGFRβ mutant mice have also kidney glomeruli that almost lack mesangial cells (Leveen et al. 1994; Soriano 1994). Additionally, placenta has a reduced number of pericytes that correlates with a reduction in fetal vessel density and increase in vessel diameter (Ohlsson et al. 1999).

Most PDGF-AA null mutants die on the tenth embryonic day (E10), but some survives up to the sixtieth postnatal day (P60) (Bostrom et al. 1996). Postnatally surviving knock-outs display a defect in alveolar formation, a reduced number of intestinal villi that have abnormal thickness and length, thin dermis, disrupted hair cycles, hypomyelination, reduction in retina astrocytes and spermatogenic arrest (Betsholtz 2004). Similar phenotypes are observed in PDGFRα knockout mice.

PDGF-CC plays a critical role in the formation of palate (Ding et al. 2004). However, the role for PDGF-DD in the development is still unclear since there are no knock-out models available so far.
3. Role of VEGF and PDGF signaling pathways in tumor progression

Tumor progression has been linked to different cell types apart from cancer cells including endothelial cells, cancer-associated fibroblasts (CAFs), pericytes and inflammatory cells (Figure 5). All of these cells send and receive pro-survival and anti-apoptotic signals to and from each other. VEGF and PDGF play a critical role in this network since both ligands and receptors are widely expressed (Figure 5).

Both VEGF and PDGF play crucial roles in cancer progression. The most important mechanisms involved in this process are autocrine stimulation of some of the tumor cells, regulation of tumor angiogenesis and regulation of interstitial fluid pressure, which will be discussed below. Targeting these pathways has already been found to have a beneficial tumor treatment effect in clinical trials.

**Figure 5.** VEGF and PDGF interplay in the tumor tissue. Arrows represent a production of PDGF (red) and VEGF (green).
3.1. Autocrine stimulation of cancer cells through VEGF and PDGF signaling pathways

Several different malignancies have been shown to be associated with mutation and activation of PDGF or VEGF signaling pathways directly on tumor cells which serves as target for anti-VEGF and anti-PDGF therapies. The amplification of the PDGFRα has been seen in glioblastoma (Fleming et al. 1992). In freshly isolated tumor cell lines, both PDGFR and PDGFs have been found to be expressed, especially PDGF-AA, PDGF-CC and PDGFRα, but also PDGF-BB, PDGF-DD and PDGFRβ (Lokker et al. 2002). Inhibition of PDGF signaling pathway slows down glioma cell growth in experimental tumors (Uhrbom et al. 2000; Lokker et al. 2002; Servidei et al. 2006). Although imatinib (see chapter 4.1.1.) as monotherapy had a minor effect, most likely due to poor penetration of the drug across the blood-brain barrier, the combination with hydroxyurea had promising results (Wen et al. 2006). In the gastrointestinal stromal tumor (GIST), PDGF signaling is activated by point mutations in the PDGFRα or in the related receptor c-Kit (Heinrich et al. 2003). Most GIST patients (80-90%) carry either PDGFRα or c-Kit mutation, and imatinib is currently used to treat these patients. The first evidence of translocation of the PDGFRβ was described in patients with chronic myelomonocytic leukemia (Steer et al. 2002). Finally, translocation of the PDGF-B gene close to the collagen gene can also occur in dermatofibrosarcoma protuberans (DFSP) (Simon et al. 1997). In carcinomas, the production of PDGF is generally thought to act in a paracrine mode; however, autocrine stimulation can also occur in some cases. In human breast carcinoma, the expression of PDGF was correlated with poor prognosis and advanced stages of tumors (Seymour et al. 1993). Increased expression of PDGF was correlated to epithelial-to mesenchymal transition (EMT) and metastasis in mammary carcinoma (Jechlinger et al. 2003).

In addition to endothelial cells, VEGFR-1 is also expressed by some tumor cells including non-small-cell lung carcinoma, melanoma, colon, and prostate cancer (Decaussin et al. 1999; Ferrer et al. 1999; Lacal et al. 2000; Fan et al. 2005). In several breast carcinoma cell lines, it was demonstrated that VEGFR-1 is expressed and stimulation with VEGF leads to increase in invasion and increased tumorgenesis (Price et al. 2001). Also, in leukemia, tumor cell growth is promoted by autocrine stimulation of VEGFR-1 (Dias et al. 2000).

3.2. Regulation of tumor angiogenesis

3.2.1. History of tumor angiogenesis research

Angiogenesis is necessary to supply solid tumors with nutrients and oxygen. Already in 1945 Algire and Chalkley observed the growth of transplanted...
mouse carcinomas and postulated that rapid tumor growth is dependent on the development of a vascular network (Algire et al. 1945). In 1968 Greenbland demonstrated that this process is mediated by factors released by the tumor cells (Greenblatt et al. 1968). However, it was Folkman who accelerated the field of anti-angiogenesis as a therapeutic option to treat cancer (Folkman 1971). He predicted that solid tumors are unable to grow beyond 1-2 mm³ without attracting new blood vessels, suggesting that tumor cells secrete diffusible angiogenic factors. Based on these predictions, he proposed a model of tumor dormancy due to blocked angiogenesis, and suggested anti-angiogenic strategy as a way to treat cancer. Also, his group isolated a serum protein at that time was termed tumor angiogenesis factor (TAF), which was an endothelial cell mitogen (Folkman et al. 1971). In 1983 Dvorak and Senger isolated a vascular permeability factor (VPF) (Senger et al. 1983) that has later on turned out to be VEGF (Keck et al. 1989) cloned by the group of Napoleone Ferrara in 1989 (Leung et al. 1989). Since then many anti-angiogenic strategies have been proposed and showed to have clinical benefits in anticancer treatments either as monotherapies or as combination therapies.

The original hope for the use of anti-angiogenic strategies in cancer therapies was to target more genetically stable cells as compared to tumor cells. This in turn would be less likely to elicit resistance or tolerance during the treatment.

3.2.2. Angiogenic switch versus tumor progression

The angiogenic switch is the transition from a pre-vascular to a vascularized tumor phenotype and is controlled by the balance between pro- and anti-angiogenic factors (Figure 6), which are secreted by tumor cells or by cells from the tumor microenvironment (e.g. stromal cell and immune cells). The evidence for the angiogenic switch and its requirement for tumor growth has been obtained by transplanting tumor cells into the cornea of rabbits (Gimbrone et al. 1974) and confirmed by implantation of tumors into chicken embryos in the chick chorioallantoic membrane (CAM) assay (Ausprunk et al. 1975). In both studies, tumor cells were implanted into avascular areas and could elicit the ingrowth of new capillaries, suggesting that tumors release factors responsible for angiogenesis. The most important are VEGF, PDGF, fibroblast growth factor (FGF), interleukin-8 (IL-8), PIGF, TGFβ, pleiotrophin, angiopoietin-1 (Ang-1) and others (Kerbel et al. 2002). Anti-angiogenic factors are either proteolytic fragments of the extracellular matrix (e.g. endostatin), cleaved derivates of plasminogen (e.g. angiotatin), or antithrombin III (Nyberg et al. 2005). The level of pro- and anti-angiogenic factors is controlled by oncogenes, tumor suppressor genes, transcription factors and environmental factors, such as oxygen and glucose supply (Gupta et al. 2003).
The initiation of the angiogenic switch starts when pro-angiogenic factors are released or anti-angiogenic factors are inhibited, resulting in endothelial cell proliferation and migration followed by vessel sprouting and tube formation. In addition, vessel intussusceptions of pre-existing vessels can occur (Burri et al. 2004). Another strategy to enhance blood supply is so called vascular co-option when tumor grows along existing blood vessels (Holash et al. 1999). A third option is vasculogenesis, which is de novo formation of blood vessels from bone marrow-derived precursor cells or formation of mosaic vessels by integrating both tumor cells, vascular cells and hematological progenitor cells into de novo vessels (Rafii et al. 2002). The last strategy is vasculogenic mimicry which is a formation of vasculogenic network from the tumor cells without involvement of vascular cells. This phenomena is observed in highly aggressive melanosomas, although the contribution of this process in the tumor progression is still not clear (Hendrix et al. 2003).

![Angiogenic switch diagram](image)

**Figure 6. The schematic illustration of the angiogenic switch.**

### 3.2.3. The concept of tumor vessel normalization

Tumor vasculature is abnormal, immature and leaky as compared to the vasculature in normal tissues. The lining endothelial cells have aberrant morphology, pericytes that support them are detached and the basement membrane is either unusually thick or absent (Figure 7) (Yuan et al. 1996; Kadambi et al. 2001; Tong et al. 2004; Winkler et al. 2004). Because of that, blood flow is inefficient and interstitial fluid pressure is increased, which causes insufficient delivery of oxygen and nutrients. Such conditions kills
many tumor cells by cutting them off from the sources of energy, but it also causes changes in the gene expression pattern of the remaining ones. In consequence, several signaling pathways are upregulated making tumor cells more resistant to harsh conditions (Bottaro et al. 2003). In the lack of oxygen, hypoxia-inducible factor (HIF) is released and stimulates the production of VEGF, that in turn promote angiogenesis. In addition, HIF induce c-Met, the receptor for hepatocyte growth factor (HGF) and increases cell motility, invasion and metastasis (Bottaro et al. 2003). Additionally, inappropriate blood supply provides a problem in anticancer therapies, since it makes the uptake of cytotoxic agents inefficient and it causes the resistance to radiotherapies due to hypoxia. Therefore, a novel concept of anti-angiogenesis, proposed by Jain (Jain 2005) is to normalize abnormal tumor vasculature to transiently improve blood vessels flow rather than inhibiting angiogenesis completely. This would be followed by functional improvements in the vasculature and improved penetration of conventional chemotherapies. Vascular normalization is also suggested to improve microenvironment that could in turn lead to increased tumor cell proliferation making them more sensitive for cytotoxic agents (Jain 2005; Willett et al. 2005; Jain et al. 2006).

![Figure 7. A schematic illustration of structural differences between normal and tumor blood vessel.](image)

Jain’s hypothesis suggests that there is a so called “normalization window” when both dose and a schedule of anti-angiogenic treatment is optimized, which can be found for individual patients. During this time period the response to radiotherapy and chemotherapy is expected to be best (Jain 2005).
3.2.4. Role of VEGF in tumor angiogenesis

Like in normal angiogenesis, VEGF is one of the most important growth factor responsible for the formation of new blood vessels in tumor angiogenesis. VEGF is expressed by most tumor cells as a consequence of genetic factors (such as activation of oncogenes or loss or mutation of tumor suppressor genes) and environmental factors such as hypoxia, low pH, inflammatory cytokines (e.g. interleukin-6), growth factors (e.g. FGF), sex hormones (both androgens and estrogens), and chemokines (e.g. stromal-derived growth factor-1, SDF-1) (Kerbel 2008). VEGF can also originate from different types of cells within the tumor mass like platelets, muscle cells, immune cells and CAFs (Fukumura et al. 1998; Liang et al. 2006; Kut et al. 2007). Endothelial cells express numerous VEGF receptors, but they produce very little or no detectable level of VEGF ligand.

Endothelial progenitor cells (EPCs) attracts interest since they represent a novel target for therapeutic intervention. They facilitate the initial establishment of tumor endothelium, control tumor growth and can determine sensitivity to chemotherapy. Also, EPCs positively correlates with invasive stages of tumors. However, their relative contribution to neovasculature formation is still debated since it varies in different tumor models and since there is no unique panel of cell-surface markers that define EPC (Patenaude et al.).

VEGF alone is not sufficient for functional new blood vessels to form. Vessel maturation and stabilization require other factors like PDGF as will be discussed below.

A study by Greenberg presented a novel role of VEGF as a negative regulator for pericyte recruitment that involves VEGFR2/PDGFRβ complex formation (Greenberg et al. 2008). He suggested, that anti-VEGF therapies not only target specifically endothelial cells on immature vessels thus resulting in more mature vascular network, but also directly mural cells and their recruitment to the vascular bed.

3.2.5. Role of PDGF in tumor angiogenesis

In the tumor mass, PDGF-BB is produced by endothelial cells, certain tumor cells and fibroblasts leading to proliferation and differentiation of mural cells expressing PDGFRβ (Figure 5) (Hellstrom et al. 1999). Tumor-derived PDGF-BB was found to be involved in the formation of tumor stroma (Forsberg et al. 1993). Overexpression of PDGF-BB in tumors leads to increase in pericyte coverage and subsequent tumor growth rate (Furuhashi et al. 2004). The origin of PDGF-dependent pericytes in the tumor is still incompletely characterized. It seems that they come from different sources; some most likely from proliferation of existing pericyte population, some from conversion of stromal fibroblasts to pericytes, and some from bone-marrow-derived precursors (Song et al. 2005; Lamagna et al. 2006). Tumor pericytes are often detached from the endothelial cells and express abnormal markers such
as alpha-smooth muscle actin (ASMA) (Morikawa et al. 2002). Different markers for pericytes are commonly used, but whether they correlate to "bona fide" pericytes remain unclear. Moreover, these markers are not pericyte-specific. ASMA is not expressed on pericytes in normal capillaries, but its expression is induced during vascular remodeling both in tumors and in retina (Morikawa et al. 2002). In addition, it is also expressed by myofibroblasts (Skalli et al. 1986). PDGFRβ and NG2 proteoglycan are expressed on pericytes in both normal vessels and in tumor vessels (Hellstrom et al. 1999; Ozerdem et al. 2002; Abramsson et al. 2003). Desmin has been used as a marker for a more differentiated pericyte phenotype (Song et al. 2005).

Some studies have indicated that PDGFRβ can be expressed in tumors on capillary endothelial cells (Hwang et al. 2003; Kim et al. 2004). However, PDGFRβ knock out studies showed that initial vascular formation during embryonal development is not disturbed, suggesting that the role of PDGF is limited to the later stages of angiogenesis (Soriano 1994). The presence of PDGFRβ on endothelial cells remains controversial and the literature is full of conflicting information. First of all, PDGFR antibodies that are so far the best tools used in immunohistochemistry, have been incompletely characterized with regard to specificity. Also, it is difficult to distinguish staining of endothelial cells and pericytes using most standard tissue analyses. It is also possible, that expression of PDGFRβ on endothelial cells described by the literature, are limited to certain types of tumors. Evidence has been presented for the presence and functional role of PDGFR on lymphatic endothelial cells in murine fibrosarcomas (Cao et al. 2004).

PDGF-CC has also been shown to be involved in tumor angiogenesis. In tumors refractory to anti-VEGF treatment CAFs were shown to stimulate secretion of PDGF-CC, which in turn stimulated tumor angiogenesis (Crawford et al. 2009). In the model of glioblastoma, PDGF-CC induced maturation of blood vessels and attenuated the response to anti-VEGF therapy (di Tomaso et al. 2009). Paracrine signaling by PDGF-AA secreted by the tumor cells recruited CAFs in xenograft studies of breast and lung carcinoma (Shao et al. 2000; Tejada et al. 2006). In another study tumor cells that were deprived from the ability to produce VEGF-A were found to compensate by secreting PDGF-AA to attract stromal fibroblasts, which in turn provide the source of VEGF-A (Dong et al. 2004). Ectopic expression of PDGF-DD also increased tumor angiogenesis which correlated to increase in tumor growth (Furuhashi et al. 2004). However, the general functional importance of this PDGF isoform is not known.
3.3. Regulation of interstitial fluid pressure

3.3.1. Definition and role of the interstitium

Interstitium is defined as the space between cells and blood or lymphatic vessels. It is composed of collagen fibers, hyaluronan, glycosaminoglycans, water, electrolytes, salts and proteins derived from plasma (Aukland et al. 1993). The ground substances are produced by mesenchymal cells, mainly fibroblasts. The interstitium serves as a transport medium for the nutrients delivered to the cells by the vascular system and for waste products that are secreted by the cells to the lymphatic network. The amount, structure and composition of the interstitium are variable in different organs and the general role is to maintain the fluid homeostasis in the organs (Wiig et al. 2003). The transcapillary flow, which is important for the fluid balance, is determined by the so called Starling forces and their relation is described by the starling equation (Aukland et al. 1993):

\[ J_v = L_p S \left[ (P_c - P_i) - \sigma (\pi_c - \pi_i) \right], \]

where \( J_v \) is the transcapillary fluid flux, \( L_p \) is a hydraulic conductivity, \( S \) is the surface area for exchange, \( [(P_c - P_i) - \sigma (\pi_c - \pi_i)] \) is the net pressure difference; \( P_c \) and \( P_i \) are the hydrostatic pressures of capillary and interstitium, respectively; \( \pi_c \) and \( \pi_i \) are the colloid osmotic pressure of capillaries and interstitium, respectively; \( \sigma \) is the plasmaprotein reflection coefficient for proteins.

The force that determines the net inward filtration is the osmotic pressure in the capillaries which under normal conditions is around 28 mmHg. The forces that determine net outward filtration are the capillary hydrostatic pressure (around 20 mmHg), the interstitial fluid osmotic pressure (about 8 mmHg) and the interstitial fluid hydrostatic pressure (around -1 to -3 mmHg). Under normal conditions there is therefore a net outward filtration pressure, which allows for the transport of nutrients to the cells (Heldin et al. 2004). However, there are variations in the velocities of the interstitial fluid (Chary et al. 1989). High molecular weight compounds like soluble proteins are mainly transported in the tissues by convection (Rippe et al. 1994), while low molecular weight compounds, such as glucose and oxygen are mainly transported by diffusion (Michel et al. 1999).

3.3.2. Mechanisms that regulate IFP in tissues

In pathological conditions, such as inflammation and burn injuries, there is an increase in net filtration pressure due to a fall in the IFP, resulting in edema formation (Lund et al. 1988). This suggests that there is an active regulation of IFP by the interstitium. One of the mechanisms is that fibroblasts exert a tension on collagen microfibrillar network of the extracellular...
matrix through collagen-binding integrins. The collagen in turn counteracts swelling of hyaluronan and proteoglycans (Wiig et al. 2003).

There are several discovered pathways responsible for the control of the IFP through fibroblasts. PDGF-BB modulates the IFP by regulating the mechanical contractile activity of fibroblasts in collagenous matrices through activation of PI3K (Clark et al. 1989; Heuchel et al. 1999). Under normal conditions tension between connective tissue cells and collagen fibers is regulated by β1 integrins (Gullberg et al. 1990; Rodt et al. 1996). They are likely to be targeted during inflammation causing a reduction in IFP and edema formation (Rodt et al. 1994). When β1 integrins are inhibited, PDGF-BB stimulates the contraction of collagen fibers through αvβ3 integrins (Figure 8) (Grundstrom et al. 2003; Liden et al. 2006).

Additionally, TGFβ and prostaglandins were also found to actively regulate the IFP. TGFβ increases the IFP by increasing the fibrosis in tumors while prostaglandins decrease the contractile activity of fibroblasts (Heldin et al. 2004).

![Diagram of collagen-fiber interactions](image)

Figure 8. Schematic illustration of the decreased compaction of the loose connective tissue upon stimulation with PDGF-BB.

3.3.3. Causes and consequences of elevated interstitial fluid pressure in solid tumors

Solid tumors often display elevated interstitial fluid pressure, compared to normal tissues. The study by Young et al. showed that the “tissue pressure” of testicular tumors was substantially elevated compared to normal tissue (Young et al. 1950). Thereafter, it has turned out that most solid tumors including breast carcinoma (Less et al. 1992; Nathanson et al. 1994), head and neck carcinoma (Gutmann et al. 1992) and colorectal carcinoma (Less et al. 1992) display increased IFP; up to 60 mmHg has been recorded in melano-
mas (Boucher et al. 1991; Curti et al. 1993). This in turn results in a net inward filtration pressure. Tumor IFP is usually uniform in the centre of the tumor and it drops gradiently in its periphery (Boucher et al. 1990; DiResta et al. 1993).

There are several reasons for higher IFP in tumors. As discussed above, tumor vessels are abnormal as compared to normal vessels. They are often leaky for macromolecules, resulting in increased tissue colloid osmotic pressure (Stohrer et al. 2000), and poorly perfused resulting in low capillary blood pressure. Blood vessel flow can also be reduced by compression by the growing tumor cells (Griffon-Etienne et al. 1999; Padera et al. 2004). In addition, tumors have greater amount of fibroblasts and denser network of collagen fibers. The importance of tumor stroma in regulation of IFP has been studied; tumor cell lines injected at diverse sites of the organs were found to give rise to divergent absolute pressure (Brekken et al. 2000). Moreover, tumor tissue is often infiltrated with immune cells that release several cytokines and growth factors that are responsible for the increased IFP (Heldin et al. 2004).

Elevated IFP forms a barrier to transcapillary transport of nutrients and oxygen, and also results in inefficient uptake of therapeutic agents (Jain 1987a; Jain 1987b). Additionally, elevated IFP has been linked to poor prognosis (Curti et al. 1993; Hagendoorn et al. 2006) and higher reoccurrence of the cancer disease in different types of tumor (Roh et al. 1991; Milosevic et al. 2001). In human melanoma xenografts, high IFP was associated with the development of pulmonary and lymph node metastases (Rofstad et al. 2002). There are also studies indicating that lowering IFP increases the efficiency of radioimmunotherapy, possibly by increasing the oxygen pressure (pO₂) in the tumor (Baranowska-Kortylewicz et al. 2005). Therefore there is a need to explore the mechanisms underlying the increase in IFP to better understand the biology of cancer. From the clinical point of view lowering of IFP is important to make anti-cancer therapies more effective.

3.3.4. Pharmacological intervention to lower IFP in solid tumors

A number of agents either delivered locally or systemically, has been shown to decrease tumor IFP. Some of them act on the microvasculature, like nicotinamide (Lee et al. 1992) and bradykinin B2 agonist (Emerich et al. 2001), whereas others affect the interstitium, like hyaluronidase (Brekken et al. 1998). TGFβ inhibitors have also been found to lower IFP by reducing relative fibrosis and normalizing tumor vasculature (Lammerts et al. 2002). The delivery of prostaglandin E (Rubin et al. 2000) or TNFα leads to lower IFP and to an increase in tumor uptake of low molecular weight compounds (Kristensen et al. 1996).
3.3.5. Lowering of tumor IFP by anti-VEGF treatment

VEGF inhibition lowers the interstitial fluid pressure by “normalizing” disorganized tumor vasculature and decreasing vessel permeability. After treatment, vessels are narrowed, a pressure gradient across the vasculature is induced and the penetration of large molecules is increased (Tong et al. 2004). This consequently causes an improvement of drug penetration in tumors and improved treatment efficiency. However it has been reported that tumor hypoxia increases as well (Franco et al. 2006). One explanation for such paradox is that a transient “vessel normalization”, proposed by Jain and described above (Jain 2005), improves the oxygenation early on during the therapy, but that later during anti-angiogenic therapy the vessels of the tumor tissues deteriorate with a concomitant hypoxia (Winkler et al. 2004). In addition it is possible that tumor cells in the presence of anti-angiogenic drug treatment may not repopulate during the brake of pulsatile chemotherapy (Franco et al. 2006) which might slow down the drug resistance and even when there is a sustained tumor hypoxia, the effects of chemotherapy can be significantly enhanced. This issue is still unclear and needs to be further investigated.

Treatment of immunocompromised mice with an antibody targeting VEGF caused a reduction in IFP of colon adenocarcinoma (LS174T) and glioblastoma multiforme (U87) tumor models (Lee et al. 2000). VEGF blockage caused an increase in the drug CPT-11 uptake in animals with adenocarcinoma (Wildiers et al. 2003). Bevacizumab, a VEGF direct antibody treatment had an effect on the reduction of tumor IFP of patients with rectal carcinoma (Willett et al. 2004).

3.3.6. Lowering of tumor IFP by anti-PDGF treatment

Since it was discovered that PDGF-BB plays an important role in the regulation of the IFP in normal tissues it was suggested to study the role of PDGF signaling on tumor models. As expected, treatment with PDGF antagonists was found to lower tumor IFP. The receptor tyrosine kinase inhibitor, imatinib, as well as PDGF-B aptamers, were found to lower IFP on KAT-4 tumors grown in immunocompromized mice (Pietras et al. 2003) and in PROb colon carcinomas grown in syngenic rats (Pietras et al. 2001). The decrease in IFP was followed by an increase in the uptake and treatment effects of chemotherapeutic agents (Pietras et al. 2002; Pietras et al. 2003). In mice bearing non-small-cell lung carcinomas, treatment with imatinib caused a reduction in the phosphorylation of PDGFRβ receptor and reduction of IFP. Additionally, tumor oxygenation was significantly improved (Vlahovic et al. 2006). In several studies, combination of anti-PDGF treatment together with radiotherapy had a beneficial effect (Russell et al. 2003; Baranowska-Kortylewicz et al. 2005; Holdhoff et al. 2005; Baranowska-Kortylewicz et al. 2007). In the LS174T colorectal carcinoma model (Baranowska-
Kortylewicz et al. 2005), as well as in the SW1990 pancreatic adenocarcinoma model (Baranowska-Kortylewicz et al. 2007), the effect was linked to an improved oxygenation due to PDGFRβ inhibition. Imatinib treatment of glioblastoma increased the cytotoxicity of irradiation through inhibition of PDGFR (Holdhoff et al. 2005). In the in vitro glioma tumor cell model, the effect of imatinib on radiosensitization was linked to reduction of Rad51 expression, which is an essential component of the DNA repair pathway and is implicated as determinant of cellular radiosensitivity (Russell et al. 2003).

The PDGF signaling pathway is crucial for maintaining the pericyte recruitment and vessel stability. Therefore it was proposed that anti-PDGF treatment might affect vessel normalization and therefore affect the IFP. However, different tumor models that display lower IFP after anti-PDGF treatment did not present any effects on the morphology of tumor vessels (Pietras et al. 2002).
4. Receptor tyrosine kinase inhibition

Traditional chemotherapies do not discriminate between dividing normal cells (e.g. bone marrow and gastrointestinal tract) and proliferating cancer cells thus leading to several toxic side effects. In contrast, targeted therapies that affect tumor-specific signaling pathways have a high specificity and are less toxic. There are several strategies to inhibit receptor tyrosine kinase signaling including antibodies, antagonistic ligands, inhibitors of protein-protein interactions and small molecule inhibitors of protein kinase activity (Bennasroune et al. 2004).

The use of small synthetic molecules has also a number of potential advantages. First of all, RTK inhibitors can block all signaling from the receptor, irrespective of the ligand concentration or type. For example, in addition to VEGF-A, VEGF-C and VEGF-D bind and activate the VEGFR-2. Therefore inhibiting signals from these additional ligands offers advantages over selective inhibition of VEGF-A like it is in the case of the VEGF-A monoclonal antibody, bevacizumab. Additionally, because of high similarity within kinase domains, inhibitors can influence other receptors within the same family. From the clinical point of view, RTK inhibitors are also practical since they are orally bioavailable. The effect of RTK inhibitors is rapidly reverted since they have a short plasma half-life, as compared to antibodies with long plasma half-life (e.g. bevacizumab, which have a plasma half life of around 3 weeks). This property is beneficial when there is severe side effects, or a need to transiently avoid problems with wound healing, e.g. in sudden requirements for surgical intervention.

RTK inhibitors may cause remission or stabilize tumor progression creating a chronic disease which is no longer life threatening. Also, side effects are minimal as compared to conventional chemotherapies. And finally synergic effects are observed when combined with chemotherapies. However, resistance to RTK inhibitors usually occurs after some time.

4.1. Receptor tyrosine kinase inhibitors-mechanism of action

The tyrosine kinase activation involves sequential events and was briefly described on the example of VEGFR (Figure 2). Small molecule inhibitors prevent ATP binding to the well-conserved ATP binding site in the kinase
domain of the receptor. Despite high similarity, discrete differences in kinase structures occur making it possible to design selective RTK inhibitors. A non-conserved hydrophobic region adjacent to ATP binding site is a major determinant of selectivity and is called “selectivity pocket”. Small-molecule inhibitors having high affinity to this region prevent the access of ATP to the catalytic site. Consequently the receptor cannot participate in phosphorylation and signal transduction.

**Table 1. VEGF and/or PDGF tyrosine kinase inhibitors that are under clinical studies.**

<table>
<thead>
<tr>
<th>Name (company)</th>
<th>Target</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>VEGFR tyrosine kinase inhibitors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTK787, Vatalanib (Novartis)</td>
<td>VEGFR (1,2)</td>
<td>(Wood et al. 2000; Morgan et al. 2003)</td>
</tr>
<tr>
<td>AZD2171 (Astra Zeneca)</td>
<td>VEGFR (1,2,3), c-kit</td>
<td>(Drevas et al. 2007)</td>
</tr>
<tr>
<td>AMG-706 (Amgen)</td>
<td>VEGFR (1,2,3), c-kit</td>
<td>(Polverino et al. 2006)</td>
</tr>
<tr>
<td>CEP-7055 (Cephalon)</td>
<td>VEGFR (1,2,3), c-kit</td>
<td>(Ruggeri et al. 2003)</td>
</tr>
<tr>
<td><strong>PDGFR tyrosine kinase inhibitors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>STI571, Imatinib/Glivec (Novartis)</td>
<td>PDGFRα/β, c-kit, c-Abl, DDR1</td>
<td>(Capdeville et al. 2002)</td>
</tr>
<tr>
<td>SU101, Leflunomide (Pfizer)</td>
<td>PDGFR, EGFR, FGFR</td>
<td>(Steeghs et al. 2007)</td>
</tr>
<tr>
<td><strong>VEGFR/PDGFR tyrosine kinase inhibitors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SU11248, Sunitinib (Pfizer)</td>
<td>VEGFR (1,2,3), c-kit, PDGFRα/β, CSF-1R, Flt-3</td>
<td>(Mendel et al. 2003)</td>
</tr>
<tr>
<td>AG-013736, Axitinib (Pfizer)</td>
<td>VEGFR (1,2,3), c-kit, PDGFRβ</td>
<td>(Rugo et al. 2005)</td>
</tr>
<tr>
<td>SU14813 (Pfizer)</td>
<td>VEGFR (1,2), c-kit, PDGFRα/β, CSF-1R, Flt-3</td>
<td>(Patyna et al. 2006)</td>
</tr>
<tr>
<td>ABT-869 (Abbott)</td>
<td>VEGFR (1,2), c-kit, PDGFRβ, CSF-1R, Flt-3</td>
<td>(Zhou et al. 2009)</td>
</tr>
<tr>
<td>OSI-930 (OSI)</td>
<td>VEGFR (1,2), c-kit, PDGFRβ, CSF-1R</td>
<td>(Garton et al. 2006)</td>
</tr>
<tr>
<td>BAY 57-9352, Telatinib (Bayer)</td>
<td>VEGFR (2,3), c-kit, PDGFRβ</td>
<td>(Eskens et al. 2009)</td>
</tr>
<tr>
<td>GW-786034, Pazopanib (Glaxo)</td>
<td>VEGFR (1,2), c-kit, PDGFRα/β</td>
<td>(Bukowski et al.)</td>
</tr>
<tr>
<td>BAY-43-9006, Sorafenib (Bayer/Onyx)</td>
<td>VEGFR (2,3), C and B-Raf, c-kit PDGFRβ, Flt-3</td>
<td>(Wilhelm et al. 2004; Clark et al. 2005)</td>
</tr>
<tr>
<td>ZK-304709 (Bayer Schering Pharma AG)</td>
<td>VEGFR (1,2,3), PDGFRβ, CDK (2,1,4)</td>
<td>(Siemeister et al. 2006)</td>
</tr>
</tbody>
</table>
4.1.1. Imatinib

Imatinib known also as Glivec or STI571 is the first tyrosine kinase inhibitor used clinically. It was originally designed to inhibit c-Abl, but later it was shown that it also targets other signaling pathways including PDGFRα/β, c-Kit, Arg (Buchdunger et al. 2000) and discoidin domain receptor 1 (DDR1) (Day et al. 2008). Imatinib gives astonishing treatment results in patients with chronic myelogenous leukemia (CML) (Capdeville et al. 2002). However, in the late stage of the disease the recurrence rate is high. It is also used to treat patients with PDGFR mutations like, the ETV6-PDGFRβ fusion protein in chronic myeloproliferative diseases (Apperley et al. 2002) or in GIST patients (Demetri et al. 2002). In DFSP, imatinib is used in cases that are not manageable with surgery alone (McArthur et al. 2005). In the model system studied with mastocytosis (Zermati et al. 2003), small cell carcinoma of lungs (Wang et al. 2000), Ewing’s sarcoma cells (Merchant et al. 2002) and colorectal carcinoma (Attoub et al. 2002) positive response to imatinib was also observed. Large advantages of the drug are good bioavailability after oral administration and presence of only mild side effects (anemia, edema, fatigue, nausea, pleuritic pain, diarrhea, granulocytopenia, and rash).

4.1.2. Vatalanib

PTK787 is a potent inhibitor targeting VEGFR-1 (Flt-1) and VEGFR-2 (FLK1/KDR). At higher concentrations it also inhibits PDGFRβ, c-Kit and c-Fms (Lin et al. 2002). In human tumor xenografts it was shown that PTK787 inhibited tumor growth what was accompanied by inhibition of tumor angiogenesis (Lin et al. 2002; Rini et al. 2005). In multiple myeloma, PTK787 directly targets tumor cell growth and survival overcoming drug resistance (Lin et al. 2002). PTK787 is being studied in mono- and combination-therapies in patients with colorectal cancer as well as liver metastases, prostate cancer, renal cancer, and refractory glioblastoma multiforme, where VEGF was overexpressed (Arora et al. 2005). The most common side effects of the drug are ataxia, vertigo, hypertension and thromboembolism.

4.2. Drug resistance and a need for combination therapies

Despite many successes in animal models with RTK inhibitors, a clinical problem is that tumors that initially respond to the treatment later on become refractory. There are several reasons that are linked to resistance. First of all cancer cells contain many genetic mutations and inhibiting only one of them might not be enough. Thus, inhibiting one of the pathways may be compensated by overexpression of another. Additionally, tumor cells can become resistant due to enhanced drug efflux by the active pumps that are located in
the cell membrane, by receptor down-regulation or through mutations in the target kinase that change the structure of the ATP binding pocket, so the inhibitor no longer fit. Moreover, cancer is a heterogeneous disease involving complex interactions between tumor cell and host cells like fibroblasts, endothelial cells, mural cells and inflammatory cells. This complexity is at the same time biologically fascinating, but clinically very disappointing since each component of the tumor mass promotes tumor progression. New insight into anti-cancer treatment is to use less specific tyrosine kinase inhibitors or to combine them with others or with conventional radio- and chemotherapies in order to target several pathways simultaneously.

4.2.1. Anti-angiogenic combination therapy targeting VEGF and PDGF signaling pathways

In animal studies, anti-VEGF treatment has been shown to inhibit tumor growth without any toxicity (Fong et al. 1999b; Drevs et al. 2000), but the results from clinical studies have not been so promising. It has turned out that tumor blood vessels are not always genetically stable as was considered before and acquired resistance is very frequent. There are several mechanisms involved in the intrinsic resistance to anti-angiogenic therapies including upregulation of pro-angiogenic signaling pathways and a selection of tumor cells towards those that can adapt to hypoxic conditions (Bergers et al. 2008). Additionally, it was observed that tumor vessels were often insensitive to VEGF targeting when they were highly covered by pericytes (Benjamin et al. 1999; Bergers et al. 2003). PDGF-BB has been claimed to protect endothelial cells from anti-angiogenic therapy as was described in the SK-NEP-1 human Wilm’s tumor xenografts model (Huang et al. 2004), the C6 rat tumor model (Erber et al. 2004) and in the mouse pancreatic carcinoma model (Bergers et al. 2003; Pietras et al. 2005). All these studies suggest a beneficial effect of combining anti-PDGF and anti-VEGF treatments in anti-cancer strategies. Additionally, tumor cells can escape from anti-VEGF treatment by upregulation of PDGF-CC by tumor stroma. This could explain the failure in using anti-VEGF as mono-treatment in certain tumor types highly infiltrated with fibroblasts and would suggest to combine it with anti-PDGF therapies.
Present investigation

Aim

PDGF and VEGF signaling pathways have been shown to be independently involved in the cancer progression by autocrine stimulation of cancer cells, regulation of tumor IFP and regulation of tumor angiogenesis.

My specific aims have been:

I. To study the effects of combination therapy targeting VEGF and PDGF signaling pathways on tumor angiogenesis and on tumor growth
II. To investigate the effects of anti-VEGF and anti-PDGF combination treatment on the tumor IFP
III. To explore the effect of anti-VEGF and anti-PDGF combination treatment on uptake and treatment efficiency of chemotherapy

Paper I

Identification of a subset of pericytes that respond to combination therapy targeting PDGF and VEGF signaling

In Paper I we evaluated the efficiency of a VEGFR inhibitor, PTK787, and a PDGFR inhibitor, STI571, used either as monotherapies or as combination therapy on the growth of two B16 mouse melanoma tumor models.

It is well known that solid tumors depend on blood vessels to supply them with oxygen and nutrients. Angiogenesis is being regulated by several growth factor receptor signals. VEGF is one of the most important growth factor and is produced mainly by hypoxic tumor cells and some inflammatory cells like macrophages. Newly formed branches of the blood vessels and also some tumor cells produce PDGF that recruit mural cells to migrate along the angiogenic sprout. Pericytes are responsible for vessel maturation, stabilization and integrity.

There have been several anti-angiogenic studies targeting VEGF signaling pathways. Although, some tumor models responded to anti-VEGF treatment, others turned out to be resistant. Further studies discovered that solid tumors were insensitive to treatment when tumor blood vessels were highly
covered by pericytes which serve a protective role towards endothelial cells. That observation made scientists target pericytes by anti-PDGF treatment to make endothelial cells more sensitive for anti-VEGF treatment. The idea of targeting both PDGF and VEGF signaling pathways was further investigated and showed to have synergistic anti-angiogenic and anti-tumor effect.

The B16 mouse melanoma tumor model with PDGF-dependent pericyte recruitment was previously established and described (Furuhashi et al. 2004). In this model, cells were transfected either with a vector containing the PDGF-B gene (B16/PDGF-BB), causing exogenous expression of PDGF-BB, or with a control vector (B16/mock). Tumors of B16/PDGF-BB cells are characterized by a faster growth rate and higher pericyte coverage and unchanged vessel density as compared to B16/mock tumor. This allows for comparing the effects of anti-angiogenic treatments on size-matched tumors that differ only in the maturity of the vasculature. B16 mouse melanoma does not express PDGF receptors so autocrine stimulation of tumor cells is excluded. Also, these tumors contain very little stroma.

In our study both tumor types were found to be significantly growth inhibited following combination therapy with 25 mg/kg of PTK787 and 100 mg/kg of STI571. The inhibition was greater in the B16/PDGF-BB tumors as compared to the B16/mock tumors. The tumor cells showed an increased rate of apoptosis and, for B16/PDGF-BB, a decreased rate of proliferation. The combination therapy induced reduction of the vessel density in B16/mock tumors and reduction of the vessel size in B16/PDGF-BB tumors indicating that pericyte-poor tumors contain vessels that are more sensitive for anti-angiogenic treatment, while pericyte-rich tumors seemed more normalized after treatment. Targeting of either VEGFR or PDGFR in B16/mock tumors also reduced the vessel length density, but without affecting the size of the vessels.

The morphological changes in the vasculature induced by combination therapy differed between pericyte-rich and pericyte-poor B16 tumor models, indicating that the amount of pericytes associated with the tumor vessels is important for the outcome of the combined anti-angiogenic targeting of endothelial cells and pericytes. Pericytes are heterogeneous populations of mural cells that are critically important for maintaining the vessel integrity. They express several markers such as PDGFRβ, ASMA, NG2 and desmin. In our study, PDGF-BB overexpression increased the number of pericytes positive for ASMA and PDGFRβ that were found to be both in close connection with endothelial cells and also detached from the vessel wall. Interestingly, PDGF-BB expression by tumor cells neither had effect on pericytes positive for desmin, nor for NG2 that were found in a close connection with endothelial cells. The treatment with combination of PTK787 and STI571 seemed to affect the proportion of detached pericytes positive for ASMA and PDGFRβ as well as smaller but significant number of NG2 positive pericytes in B16/PDGF-BB tumor. In the B16/mock model, treatment with combina-
tion of STI571 and PTK787 targeted only the population of PDGFRβ positive pericytes.

Interestingly, desmin-positive pericytes that were found to be in a close contact with endothelium did not respond to the combination treatment in either model. Desmin was previously found to require a close cell-to-cell contact with endothelial cells to be expressed on pericytes. The reason for the inability of desmin positive pericytes to respond to a treatment remains to be elucidated, but it seems that endothelial cells and pericytes in a close contact send growth factors and survival signals to each other. Finding out the mechanism involved in this resistance could further improve treatment efficiency in anti-angiogenic therapies at least in some tumor types.

In summary, the study demonstrates that PDGF-BB protects endothelial cells from anti-VEGF therapy and that combination of PTK787 and STI571 treatment inhibits tumor growth, which is accompanied by a reduction of a specific subset of pericytes.

Paper II

Combined anti-angiogenic therapy targeting PDGF and VEGF receptors lowers the interstitial fluid pressure in a murine experimental carcinoma

The paper describes experiments performed on KAT-4 tumors to investigate the effects of combination treatment with the PDGF receptor tyrosine kinase inhibitor, STI571, and a VEGF tyrosine kinase inhibitor, PTK787, on the tumor IFP.

Solid tumors display an increased IFP, which causes a problem in anticancer treatment since it makes the uptake of cytotoxic agents inefficient. Also, high IFP has been linked to poor clinical outcome and higher recurrence of the cancer disease. Previous studies have described that both VEGF and PDGF participate in the regulation of tumor IFP independently. STI571 reduces IFP presumably by relaxation of connective tissue cells. VEGF signaling was shown to be critical for vascular density and vessel leakiness that subsequently affects IFP.

The chosen model, KAT-4, is a well established model for studies on regulation of IFP by PDGFR inhibition. KAT-4 is highly infiltrated with fibroblasts, a target for PDGF, and it contains vasculature as a target for VEGF.

The IFP was measured using wick-in-needle technique (Rubin et al. 2000; Pietras et al. 2001). This method allows for stable and continued measurement of the pressure using a needle filled with nylon floss and saline that is inserted into the tumor and connected to a transducer. To determine differences in the extracellular fluid volume following different treatments, the extracellular volume (ECV) was measured by the dilution principle using
radiolabelled isotopes (Salnikov et al. 2006) that were given after functional nephrectomy. Vessel leakiness was quantified by injecting Evans blue into the tail vein, followed by extraction of the dye from the tumor and quantification of the amount in a spectrophotometer (Gratton et al. 2003).

As expected, both STI571 and PTK787 reduced IFP also in this model. Two days of treatment with STI571 alone followed by two days of combination treatment (named short term combination treatment) had an additive effect on the lowering of tumor IFP. Unexpectedly, combining these two agents for four days (long term combination treatment) failed to lower tumor IFP. The explanation for this observation is not known, but could be related to transient vessel normalization followed by vessel deterioration after longer exposure to the VEGFR tyrosine kinase inhibitor. Even though both combination treatments lowered vessel leakiness, the anti-angiogenic effect reflected by the number of vessels was significantly reduced only in short, but not long term combination setup. Also pericyte coverage was smaller after short, but not long term combination treatment as compared to monotherapies.

The effects of the inhibitors on the therapeutic efficiency of Taxol, a common cytotoxic agent used in chemotherapies were investigated. STI571 had an additive effect on tumor growth when given in combination with Taxol. This was explained by the concomitant increased of extracellular fluid volume (ECV), which in turn is known to increase hydraulic conductivity and consequently transport of molecules. Despite having an additive effect in decreasing tumor IFP, the combination therapy did not further enhance the effect of either of the kinase inhibitor alone. Moreover, ECV was not increased following the combination treatment. This could be due to that the decreased vessel leakiness prevents an increase in the ECV and thereby fluid convection.

In summary, we have shown that combination therapy targeting PDGFRs and VEGFRs has an additive effect on lowering the IFP, but the timing of the treatment appears to be critical. However, there was no additional benefit of the combination therapy on the Taxol treatment as compared to monotherapies.

**Paper III**

**Combination of STI571 and PTK787 improves treatment efficiency of Taxol in B16/PDGF-BB mouse melanoma tumors**

The study in Paper III follows up the study from Paper I, where it was shown that combination of anti-PDGF and anti-VEGF treatment using PTK787 and STI571 had an effect on tumor volume and vascular parameters in B16/PDGF-BB mouse melanoma. Here, we asked whether this anti-angiogenic treatment effect will improve the treatment efficiency of Taxol.
Taxol alone did not affect B16/PDGF-BB tumor volume, neither given at a low dose (5 mg/kg) nor at high dose (20 mg/kg). Importantly, only the combination of PTK787/STI571 significantly improved the treatment effect of Taxol already at 5 mg/kg. Additional in vitro studies showed, that STI571 sensitized tumor cells to Taxol. However, STI571 alone did not improve treatment efficiency of Taxol in vivo. There is a possibility that combination of PTK787 and STI571 targeted blood vessels, causing transient improvement of the uptake of Taxol and STI571. However, it is difficult to estimate the relative contribution of the anti-angiogenic and sensitizing effect of PTK787/STI571 treatment on the overall outcome of the Taxol therapy. Even though we did not see the increase uptake of radiolabelled Taxol following the treatment with STI571 and PTK787 at the day of sacrifice, one cannot exclude the possibility that the uptake was improved earlier on during the treatment.

STI571 targets PDGFR, but also other kinases including Abl kinase, the stem cell factor receptor c-Kit and DDR1. To determine the molecular targets for the effect of sensitization to Taxol, in vitro growth assays were performed. It was previously shown that B16 melanoma does not express detectable levels of PDGFR and does not respond to PDGF in vitro. To look specifically at Abl, an Abl specific inhibitor, AG956, was used and was demonstrated to have no effect on cell proliferation. Since the B16/PDGF-BB cells express c-Kit, the observed effect could be due to c-Kit inhibition which is an important receptor in melanocytes and possibly also in melanomas, but this eventuality needs to be further investigated.

The sensitizing effect of STI571 in vitro was not observed in B16/PDGF-BB in combination with another cytotoxic agent 5-Fluorouracil (5-FU) that has a different mechanism of action as compared to Taxol. 5-FU blocks synthesis of the pyrimidine thymidine, which is a nucleotide required for DNA replication, while Taxol is a microtubule targeting agent. This suggests that the sensitizing effect could be related to the targeting of microtubules.

The present data shows that combination of two tyrosine kinase inhibitors, STI571 and PTK787, increases the effects of Taxol, which could provide a novel strategy for melanoma treatment that is refractory to conventional chemotherapies.
Future perspectives:

It has been suggested that anti-angiogenic therapy “normalizes” disorganized tumor vasculature and might be beneficial in combination with conventional chemotherapies. In Paper I, we found that combination of anti-PDGF and anti-VEGF treatment had an additive anti-angiogenic and anti-tumor effect in B16/PDGF-BB melanoma cells. In Paper III, we investigated whether the observed anti-angiogenic effect improved the uptake and treatment effect of Taxol. The chosen setup had a remarkable treatment effect. However, cells grown in vitro are sensitized to Taxol when treated with STI571 alone, indicating that STI571 has a direct effect on the tumor cells. To determine the contribution of a targeting of the vasculature on the effect of Taxol, a cytotoxic agent that does not sensitize cells in vitro should be used. 5-FU would be a good candidate since B16/PDGF-BB cells are not sensitive for this agent in vitro in combination with STI571 and/or PTK787. It would be important to determine the mechanism underlying the sensitizing effects of STI571 to Taxol. c-Kit seems to be the potential target of STI571, since B16 cells express this receptor, and since most of other known targets for STI571 were excluded. However, to clearly answer this question it would be important to knock-down c-Kit in B16 melanoma as well as other cell types that express c-Kit, using shRNA and repeat the in vivo experiment. In addition, it would be worth knowing whether the B16 cell line secretes the ligand for c-Kit, SCF.

The mechanisms making the tumors resistant and refractory to anti-cancer strategies are still lacking full explanation. We need to understand better these mechanisms and establish biomarkers to be able to select patients for different treatments in a better way. Clinical experience indicated that designing treatments that not only target the population of the cancer cells, but also the tumor microenvironment could be beneficial in cancer therapy. One way to achieve that is to use broad spectrum inhibitors, or to combine signal transduction inhibitors with cytotoxic therapies.

Based on our finding that well-differentiated pericytes are less responsive to anti-angiogenic treatment it would be of importance to investigate the mechanism underlying pericyte recruitment, proliferation and differentiation, as well as finding out the mechanism that protects well-differentiated pericytes from anti-angiogenic combination treatment. Furthermore, it will be interesting to determine to what extent the amount of pericytes and their degree of differentiation on tumor vessels improves their function and also to
examine whether combination treatment targeting both pericytes and endothelial cells induces vessel regression.

In Paper II, combining anti-PDGF and anti-VEGF treatment had an additive effect on the lowering of IFP in KAT-4 tumors, but only when administered according to a certain schedule. It would be important to find out the molecular mechanisms underlying differences in the outcome between shorter and longer exposure of the inhibitors.

Interestingly, further decrease in the IFP, as a result of combining anti-VEGF and anti-PDGF treatment, did not translate into treatment effect of Taxol, which was surprising and the reason for this need to be investigated. We need to find out if decreased vessel leakiness prevents an increase in fluid convection. It is possible, since STI571 treatment was the only one that increased ECV, but did not correct vessel leakiness. We also need to determine differences between the tumor and normal tissues that cause increase in the ECV following short term combination treatment in the skin but not in the tumor. More studies are needed to determine the optimal timing of the delivery of inhibitors to lower IFP, and increase the fluid convection to determine how this affects the treatment with chemotherapeutical drugs.

Since both VEGF and PDGF play roles in lymphangiogenesis and since lymphatic vessels are involved in the regulation of IFP by removing the fluid outside of the tissue, it will be important to investigate whether targeting of VEGFRs and PDGFRs affect the function and morphology of lymphatic vessels. This might not only have an impact on the IFP, but also on the metastatic properties of the tumors.

Activation of PI3K is important for PDGF-mediated increase in IFP in the skin. Therefore, we will study the mechanism underlying regulation of IFP in tumors using transgenic mice with mutations in the PI3K-binding site of the PDGFRβ. Tumor IFP and tumor uptake of Taxol following STI571 treatment will be measured.
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