Università degli Studi dell'Insubria

Scuola di Dottorato in Scienze Biologiche e Mediche
Dottorato di Ricerca in Biologia Cellulare e Molecolare
XXIII ciclo

PhD Thesis:

Cell delivery of Met docking site peptides inhibit angiogenesis and vascular tumor growth

Supervisor:
Dr. Adriana Albini

Candidate:
Dr. Anna Rita Cantelmo
Matricola: 708203

Academic Year 2009-2010

List of abbreviations

17-AAG	17-allylamino-17-demethoxygeldanamycin
8-Cl-Ado	8-clhoro-adenosine
Antp	Antennapedia homeodomain
CML	Chronic myelogenous leukaemia
CPPs	Cell-penetrating peptides
ECM	Extracellular Matrix
ECs	Endothelial Cells
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EPCs	Endothelial Precursors Cells
FAM	5-(and-6)-carboxyfluorescein
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FGFs	Fibroblast Growth Factors
HGF	Hepatocyte Growth Factor
HIF	Hypoxia-inducible Transcription Factor
HIV	Human Immunodeficiency Virus
HUVEC	Human Umbilical Vein Endothelial Cells
Met	Mesenchimal Epithelial Transition, HGF receptor
MRS	Met related sequence
MTT	[3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide]
NSCLC	Non-small-cell lung cancer
OS	Overall survival
PBS	Phosphate- buffered saline
PDGFRs	Platelet-derived Growth Factor Receptors
PDGFs	Platelet-derived Growth Factors
PEI	Polyethyleneimine
PFS	Progression free survival
PlGF	Placental Growth Factor
PTDs	Protein transduction domains
RCC	Renal cell carcinoma
RTKIs	Tyrosine kinase inhibitors
SFM	Serum free medium
SH2	Src homology-2
SMCs	Smooth Muscular Cells
TGF-β	Trasforming Growth Factor-β
TNF-α	Tumor necrosis factor−α
VEGFRs	Vascular Endothelial Growth Factor Receptors
VEGFs	Vascular Endothelial Growth Factors

INDEX

ABSTRACT

Angiogenesis is a very complex biological process that drives the formation of new blood vessels starting from pre-existing one. In adulthood, it occurs both in physiological and pathological processes. Pathological angiogenesis is involved in several diseases, including cancer. In the last decade, the concept of anti-angiogenic therapy has been developed as a strategy to treat cancer inducing the inhibition of new vessels that provide sustenance to the tumor. Recently, Met receptor and its ligand HGF (Hepatocyte Growth Factor) has been proposed as target for the anti-angiogenic therapy.

HGF and its receptor Met are responsible for a wide variety of cellular responses, both physiologically during embryo development and tissue homeostasis, and pathologically, particularly during tumor growth and dissemination. In cancer, Met can act as an oncogene on tumor cells, as well as a pro-angiogenic factor activating endothelial cells and inducing new vessel formation. Molecules interfering with Met activity could be valuable agents for anti-cancer treatment simultaneously inhibiting oncogenesis and angiogenesis.

The aim of this study has been the identification of peptides able to interfere with HGF-induced angiogenesis.

We synthesized three different peptides containing the Met docking site fused to the internalization sequences of Antennapedia homeodomain or of Tat transactivation domain which were used to deliver peptides into living cells.

In vitro we treated human endothelial cells with peptides and we quantified proliferation. We then evaluated the ability of peptides to interfere with HGF-induced migration, invasion and morphogenesis of

endothelial cells. By western blotting analysis we investigated whether these peptides affect Met activation and downstream signalling. *In vivo* we performed matrigel sponge assay and Kaposi's sarcoma xenograft to test the peptides efficacy on angiogenesis and tumor growth.

We observed that in endothelial cells internalized peptides inhibited ligand-dependent cell proliferation, motility, invasiveness and morphogenesis *in vitro,* to an even greater extent and with much less toxicity than the Met inhibitor PHA-665752, which correlated with interference of HGF-dependent downstream signalling, as shown by reduction in ERK1/2 activation (Cantelmo *et al.*). *In vivo*, the peptides inhibited HGF-induced angiogenesis in the matrigel sponge assay and impaired xenograft tumor growth and vascularization in Kaposi's sarcoma (Cantelmo *et al.*).

These data show that interference with the Met receptor intracellular sequence impairs HGF-induced angiogenesis, suggesting the use of anti-docking site compounds as a therapeutic strategy to counteract angiogenesis in cancer as well as in other diseases.

1. Introduction

1.1. Angiogenesis

Blood vessels are essential for development and reorganization of tissues in the organisms. They transport gases, liquids, nutrients, signalling molecules and circulating cells between tissues and organs, removing the catabolic products. This task is carried out by the blood vessels and the lymphatic vessels, which internal walls are formed by endothelial cells (ECs). Both endothelial networks are essential for homeostasis in the healthy organism (Rocha and Adams, 2009). The vascular system is generated by two different processes: vasculogenesis and angiogenesis (Figure 1). Vasculogenesis refers to the formation of blood vessels by endothelial cell precursors (EPCs), the angioblasts, deriving from the hemangioblasts (Risau and Flamme, 1995). The juvenile vascular system then evolves from the primary capillary plexus by subsequent pruning and reorganization of endothelial cells in a process called angiogenesis (Risau, 1997).

Nature Reviews Cancer 3, 411-421 (June 2003)

Figure 1: *Origin of endothelial cells and assembly of the vasculature*

Recent studies suggest that the incorporation of bone marrow-derived endothelial progenitor cells (EPCs) in the growing vessels complements the sprouting of resident endothelial cells (Asahara *et al.*, 1997; De Palma *et al.*, 2003; Rafii *et al.*, 2002).

During development hemangioblasts, a mesodermal common precursor of endothelial and hematopoietic stem cells (HSCs), aggregate to form the blood islands. These structures proliferate and migrate to form a network of tubular structures characterized by two layers: the internal layer becomes the endothelial coating of the nascent vessels, while the external layers differentiate to smooth muscular cells (SMCs) or pericytes. Between them there is the basal lamina rich in collagen. The fusion of blood islands forms primitive vascular plexus, in which the venous and arterial systems are already distinct (Risau and Flamme, 1995). The vascular plexus is immature and fragile and it needs the angiogenic and arteriogenic process to be stabilized.

Angiogenesis is the formation of new blood vessels starting from pre-existing ones. It is a complex process divided in different phases. The first step consists in the increase of vascular permeability of pre-existing vessels. This process requires the extracellular matrix (ECM) breakdown by proteinases, including plasminogen activator, metalloproteinases, heparinases, tryptates and cathepsyns (Alexander and Elrod, 2002). The ECs can migrate to the sites where angiogenesis is required, where they proliferate and differentiate. In the second phase there is a block of ECs migration and proliferation processes occurred to form a new network of vessels. Hypoxia is an important stimulus for expansion of the vascular bed, triggering vessel growth by signalling through hypoxia-inducible transcription factors (HIF) (Safran and Kaelin, 2003). HIFs up-regulate many angiogenic genes, such as VEGFs (Vascular Endothelial Growth

Factors), angiopoietins, FGFs (Fibroblast Growth Factors) and related receptors (Pugh and Ratcliffe, 2003). These stimuli regulate the formation of a mature vessel network, that is stabilized by the addiction of several layers of SMCs (Carmeliet, 2003).

Vascular endothelium is versatile, has multiple functions and activities of synthesis and metabolism. It is dynamic and actively takes part in the interactions between blood flow and tissue. In fact, it acts as a semipermeable membrane that regulates molecular exchanges, modulates vascular tone and blood flow, and regulates the immune response by controlling, for example, leucocyte adhesion.

1.1.1 Pathological angiogenesis

Judah Folkman described several years ago the association between growing solid malignant tumors and new vessel growth, proposing that anti-angiogenesis might represent a therapy for solid tumors (Folkman, 1971). Since then, much effort has been done in the elucidation of the mechanisms of angiogenesis.

Pathological angiogenesis is characterized by an excessive or an insufficient vessels formation. Deregulated vessels growth contributes to the pathogenesis of many disorders, some quite unexpected. Indeed, a long list of disorders is characterized or caused by excessive angiogenesis. Historically the best known are cancer, psoriasis, arthritis and blindness, but many additional common disorders such as obesity, asthma, atherosclerosis and infections disease are included and the list is still growing (Table 1). In addition, insufficient vessel growth and abnormal vessel regression not only causes heart and brain ischemia, but can also lead to neurodegeneration, hypertension, pre-eclampsia, respiratory distress, osteoporosis and other disorders (Table 2). However

the vast importance of angiogenesis as possible therapeutic target has been the driving force for the elucidation of its molecular mechanisms.

Organ	Diseases in mice or humans
Numerous organs	Cancer, infectious diseases, autoimmune disorders
Blood vessels	Vascular malformation, cavernous hemangioma, atherosclerosis, transplant arteriopathy
Adipose tissue	Obesity
Skin	Psoriasis, warts, allergic dermatitis, scar keloids, pyogenic granulomas, Kaposi sarcoma in AIDS patients
Eye	Persistent hyperplastic vitreous syndrome, diabetic retinopathy, retinopathy of prematurity, choroidal neovascularization
Lung	Primary pulmonary hypertension, asthma, nasal polyps
Intestines	Inflammatory bowel and periodontal disease, ascites, peritoneal adhesions
Reproductive system	Endometriosis, uterine bleeding, ovarian cysts, ovarian hyperstimulation
Bone, joints	Arthritis, synovitis, osteomyelitis, osteophyte formation

Modified from Nature Medicine 9(6):653-660 (June 2003)

Table 1: *Diseases characterized or caused by abnormal or excessive angiogenesis*

Organ	Disease in mice or humans
Nervous system	Alzheimer disease, amyotrophic lateral sclerosis, diabetic neuropathy, stroke
Blood vessels	Atherosclerosis, hypertension, diabetes
Gastrointestinal system	Gastric or oral ulcerations, Chron disease
Skin	Hair loss, skin purpura, teleangectasia and venous lake formation
Reproductive system	Pre-eclampsia, menorrhagia
Lung	Neonatal respiratory distress, pulmonary fibrosis, emphisema
Kidney	Nephropathy
Bone	Osteoporosis, impaired bone fracture healing

Table 2: Diseases characterized or caused by insufficient angiogenesis or vessel regression

Application of specific compounds that may inhibit or induce the creation of new blood vessels in the body may help to combat such diseases. The last two decades have seen an explosive progress in our understanding of the angiogenesis pathways underlying growth and differentiation of blood vessels. On the basis of these results several anti-angiogenic therapies have been developed with the aim to modulate pathological angiogenesis (Carmeliet, 2003).

1.1.2 Tumor angiogenesis and the angiogenic switch

The vasculature is usually quiescent in the adult life and occurs only in few tissues including female reproductive organs, organs that are undergoing physiological growth or injured tissue (Hanahan and Folkman, 1996). The formation of a tumor-associated vasculature, a process referred to as tumor angiogenesis, is essential for cancer progression, as it permits tumor expansion, favours local invasion and

provides a route for metastatic dissemination (Folkman, 1972). Studies suggest that if the vascularisation of tumors can be blocked, the tumor mass will be limited in size. Further, the acquisition of the angiogenic phenotype coincides with conversion to malignancy (Ferrara, 2000b).

Figure 2: *The classical angiogenic switch*

The angiogenic switch can occur at different stages of the tumor progression pathway and it depends on the nature of tumor and its microenvironment (Figure 2). The switch begins with perivascular detachment and vessel dilatation, followed by angiogenic sprouting, new

vessel formation and maturation, and the recruitment of perivascular cells (Bergers and Benjamin, 2003).

Tumor vessels display a different structure, easy to discriminate from the normal vessels (Pasqualini *et al.*, 2002). Vascular structures are irregular and disorganized: they have an irregular diameter, casual branches, are more permeable, hemorrhagic; the blood flow is altered and may show stasis or invert flow direction. An aberrant architecture leads to an altered functionality (Bergers and Benjamin, 2003). Vessel walls are thinner and not suitable to the lumen size, often fenestrated, thereby increasing permeability to macromolecules and cells that can pass through it. The transport of fluids and molecules into the interstitium is tightly modulated by interstitial compartment's biological and physical properties and by the physical and chemical properties of the molecules. The composition of interstitial compartment within neoplastic tissues is significantly different from normal tissues: it presents wide interstitial spaces, high collagen concentration, low proteoglycan and hyaluronic acid levels, high macromolecule diffusion coefficients and lacks a functional lymphatic network leading to a high interstitial pressure. The high interstitial fluid pressure affects the normal hydrostatic gradient that reduces convective movements, thus delaying macromolecule passage trough fenestrations (Jain, 1999). This unusual behaviour of fluid movement in and out of tumor blood vessel reflects the particular wall composition (McDonald and Baluk, 2002). The endothelial cells do not constitute a normal monolayer, but have an irregular shape, are disorganized, accumulate in multilayer and are weakly bound to each other. Recruited pericytes and the basal membrane are disorganized and weakly associated to endothelial cells (Morikawa *et al.*, 2002).

Weak interactions favour the destabilized state of vessels which are thus prone to angiogenic sprouting. Tumor vessels are in fact immature, since maturation would lead to a quiescent status. This underlies the conventional description of tumor vessels as constantly bleeding. Immunoistochemestry and ultrastructural analyses showed that tumor cells may be involved in blood vessel architecture participating in vessel lining and creating a mosaic vessel (Folberg and Maniotis, 2004; Zhang *et al.*, 2007).

The abnormal features of the tumor vasculature are belived to result from the alteration of the angiogenic balance between pro- and anti-angiogenic signals (Bergers and Benjamin, 2003). During normal physiological angiogenesis, new vessels rapidly mature and become stable. By contrast, tumors lose the appropriate balances between positive and negative controls, resulting in a disproportionate expression of angiogenic cytokines and inhibitors. A classical model of the regulation of angiogenesis is illustrated by a scale laden with anti-angiogenic molecules on one side and pro-angiogenic molecules on the other (Bergers and Benjamin, 2003). Induction of the angiogenic switch depends on how heavily that balance tips in favour of pro-angiogenesis. Tumor is one of the best known condition in which angiogenesis is switched on. Several studies demonstrate that tumor cells induce angiogenesis through the secrection of pro-angiogenic factors. Moreover, the inhibition of these factors may cause a decrease of tumor vascularization and consequent reduction of tumor growth (Ribatti *et al.*, 2007). The vascular endothelial growth factors, in particular VEGF-A, have been shown to play a central role in this process (Ferrara, 2000a).

1.1.3 Angiogenic growth factors and their receptors

New vessel growth and maturation are highly complex and coordinated processes requiring the sequential activation of a series of receptors by numerous ligands in endothelial and mural cells (Carmeliet, 2003; Jain, 1999). Many evidences indicate that tumor cells secrete pro-angiogenic growth factors that bind to receptors on dormant endothelial cells (ECs), leading to vasodilation and an increase in vessel permeability (Bergers and Benjamin, 2003; Carmeliet, 2003). The growth factors can also act on more distant cells recruiting bone marrow-derived precursor ECs and circulating ECs to migrate to the tumor vasculature (Bergers and Benjamin, 2003; Carmeliet, 2003).

Many potential angiogenic factors have been described over the past two decades (Klagsbrun, 1991; Yancopoulos *et al.*, 2000) and many evidences indicate how vascular endothelial growth factors (VEGFs) is an important regulator family for angiogenesis (Ferrara, 2002).

The VEGF family comprises seven proteins that are designated VEGF-A (Ferrara *et al.*, 2003), VEGF-B (Stein *et al.*, 1998) and PlGF (Placental Growth Factor), (Maglione *et al.*, 1991) involved in angiogenesis, VEGF-C (Barleon *et al.*, 1997; Gerber *et al.*, 1997), VEGF-D (Hood *et al.*, 1998; Waltenberger *et al.*, 1996) that take place in lymphagiogenesis, VEGF-E (Meyer *et al.*, 1999; Ogawa *et al.*, 1998) and VEGF-F (Suto *et al.*, 2005) recently isolated which biological function are not well known. The VEGFs exert their biologic effect through the interaction with specific tyrosine kinases receptors that are designed VEGFR-1 (also known Flt-,fms-like tyrosine kinase) (Masuda *et al.*, 1990), VEGFR-2 (named KDR (kinase domain receptor) in human and Flk-1 (Fetal liver kinase-1) in mouse) (Matsumoto and

9

Claesson-Welsh, 2001), VEGFR-3 (also known Flt-4) (Pajusola *et al.*, 1994). VEGF-A binds to both receptors Flt-1 and KDR/Flk-1 inducing vasculogenesis and angiogenesis. In contrast, PlGF and VEGF-B bind exclusively to Flt-1. PlGF acts on a wide range of cell types, stimulating the growth, migration and survival of endothelial cells (Adini *et al.*, 2002; Carmeliet *et al.*, 2001), increasing the proliferation of fibroblasts and smooth-muscle cells (Bellik *et al.*, 2005), inducing vasodilatation and stimulating collateral vessel growth (Bellik *et al.*, 2005). It also promotes the recruitment and maturation of angiogenesis- competent myeloid progenitors to growing sprouts and collateral vessels (Hattori *et al.*, 2001).

Many other regulatory and signalling molecules govern the angiogenesis process (Ribatti *et al.*, 2000).

The family of platelet-derived growth factors (PDGFs) and its receptors PDGFR-α and PDGFR-β are involved in vessel maturation and the recruitment of pericytes (Lindahl *et al.*, 1997), stimulating angiogenesis *in vivo* (Oikawa *et al.*, 1994; Risau *et al.*, 1992). PDGF is expressed by ECs and generally acts in a paracrine manner, recruiting PDGFR-expressing cells, particularly pericytes and smooth muscle cells to the developing vessels (Andrae *et al.*, 2008).

The mammalian fibroblast growth factors (FGFs) and their receptor are widely expressed and present on most cell types, in which they act through a wide range of biological roles (Cao *et al.*, 2008). In EC culture and animal models they lead to angiogenesis promoting the proliferation, migration, and differentiation of vascular ECs (Abraham *et al.*, 1986; Maciag *et al.*, 1984).

The EGF/EGFR (epidermal growth factor/epidermal growth factor receptor) system plays an indirect role in angiogenesis up-regulating the

production of pro-angiogenic factors such as VEGF. Activation of EGFR has been linked to angiogenesis in xenograft models in addition to metastasis, cell proliferation, survival, migration, transformation, adhesion, and differentiation (Perrotte *et al.*, 1999; Yarden and Sliwkowski, 2001).

Transforming growth factor-β (TGF-β) and corresponding receptors participates in angiogenesis, cell regulation and differentiation, embryonic development, and wound healing (Blobe *et al.*, 2000). TGF-β is believed to have both pro-angiogenic and anti-angiogenic properties, depending on the levels present. Low levels of TGF-β contribute to angiogenesis by up-regulating angiogenic factors and proteases, whereas high doses of TGF-β stimulate basement membrane reformation, recruit smooth muscle cells, increase differentiation, and inhibit EC growth (Carmeliet, 2003).

Hepatocyte growth factor (HGF) is a potent mitogenic, motogenic and morphogenic factor with angiogenic properties. The HGF/Met signalling directly or indirectly stimulate endothelial cells by activating angiogenic pathways (Dong *et al.*, 2001; Sengupta *et al.*, 2003), regulating other angiogenic factors (Zhang *et al.*, 2003) or mobilizing endothelial progenitor cells (Schroder *et al.*).

Because growth factors stimulate ECs, leading to angiogenesis, the growth factors, receptors, and subsequent signalling cascades make for promising targets in angiogenesis inhibition. Significant progress in targeting these pathways has been made, and several drugs have been approved by the Food and Drug Administration (FDA) or are currently in clinical development.

1.1.4   Modern approach in angiogenesis inhibition

The inhibition of angiogenesis and vascular targeting has been in the last decade the focus of new treatment strategies against the cancer. Numerous new substances, such as recombinant proteins and monoclonal antibodies, synthetic or natural origin compounds, were assessed as angiogenesis inhibitors and evaluated in clinical trials for safety, tolerance, and efficacy. Some of these molecules have already been approved for various indications, such as the VEGF neutralizing antibody Bevacizumab (Avastin) currently tested in several hundred clinical trials in a variety of different tumor types (Database of Current Clinical Trials, 2009). Bevacizumab is a humanized monoclonal antibody that binds to VEGF-A, preventing it from binding to receptors and activating signalling cascades that lead to angiogenesis. The use of Bevacizumab in combination with irinotecan, fluorouracil (5-FU), and leucovorin in patients with metastatic colorectal cancer significantly increased the progression free survival (PFS), as well as the median overallsurvival (OS) (Hurwitz *et al.*, 2004). Bevacizumab did not provide any benefit for patients with metastatic breast cancer when used in combination with capecitabine (Miller *et al.*, 2005), whereas increased the PFS and OS in combination with paclitaxel (Miller *et al.*, 2007). However, there was still no significant increase noted in OS, as had been observed with colorectal cancer (Hurwitz *et al.*, 2004) and non-small-cell lung cancer (NSCLC) (Johnson *et al.*, 2004; Sandler *et al.*, 2006). Actually Bevacizumab is approved for various indications in colorectal cancer, NSCLC, breast cancer, renal cell carcinoma (RCC), and glioblastoma (Cook and Figg).

Other anti-angiogenic drugs targeting multiple tyrosine kinase receptors, defined tyrosine kinase inhibitors (RTKIs), have been approved (Cook and Figg). The small-molecules Sunitinib and Sorafenib, are tyrosine kinase inhibitors targeting the VEGF receptor, primarily VEGFR-2, and have shown clinical efficacy in diverse cancer types (Gan *et al.*, 2009; Wilhelm *et al.*, 2008). Both drugs have shown benefit in patients with renal cell cancer (Escudier *et al.*, 2009; Motzer *et al.*, 2006). Sorafenib inhibits Raf serine kinase and has been approved for treatment of hepatocellular cancer (Llovet *et al.*, 2008).

Erlotinib (OSI-774) is an oral inhibitor of the EGFR/HER1 RTK that is approved by the FDA for NSCLC patients who have failed chemotherapy (Shepherd *et al.*, 2005) and for pancreatic cancer patients when used in combination with gemcitabine (Moore *et al.*, 2007).

Imatinib (STI571) inhibits the cytoplasmic and nuclear protein tyrosine kinase Abl, as well as the RTK PDGFR and c-kit and has been used extensively in the treatment of chronic myelogenous leukemia (CML) (Mahon *et al.*).

Additional RTK inhibitors are currently in clinical trials for a variety of cancers (Heath and Bicknell, 2009) (Table 3).

While these drugs have provided new ways to treat cancer, the survival benefits of these treatments are relatively modest, further, they are costly and can have toxic side effects (Eskens and Verweij, 2006; Kerbel, 2008; Verheul and Pinedo, 2007). Intrinsic and acquired resistance to anti-angiogenic drugs are becoming clinically significant problems; pre-clinical studies have begun to shed light on potential mechanisms of resistance. Murine tumor cell lines intrinsically resistant to anti-VEGF antibodies have been shown to be colonized by bone marrow derived myeloid-suppressor cells *in vivo* (Shojaei *et al.*, 2009). These myeloid

cells then make an alternative angiogenic factor, Bv8 (Shojaei *et al.*, 2009), which circumvents the VEGF blockade. Moreover, when anti-VEGF sensitive tumor cells are mixed with myeloid cells from treatment resistant tumors, these myeloid cells confer resistance (Shojaei *et al.*, 2009). Acquired resistance to VEGF pathway inhibitors can be caused by the redundancy of angiogenesis stimulators. An example is up-regulation of the angiogenesis stimulator basic fibroblast growth factor (bFGF) within the tumor after treatment with anti-VEGF therapy, which may also act synergistically with other angiogenic factors such as PDGF isoforms (Kerbel, 2008). Disruption of other angiogenesis-associated pathways may paradoxically enhance angiogenesis itself (Reynolds *et al.*, 2009). Recent studies have suggested that hypoxia induced by VEGF blockades enhances tumor cell migration, invasion and metastatic dissemination (Ebos *et al.*, 2009; Paez-Ribes *et al.*, 2009) by up-regulating activity of the HGF/Met pathway in tumor cells, which subsequently (Pennacchietti *et al.*, 2003). These observations underline the importance of finding new agents able to modulate pathological neo-angiogenesis through selective pathways. Recently, the HGF/Met axis has been proposed as a target for anti-angiogenic therapy (You and McDonald, 2008).
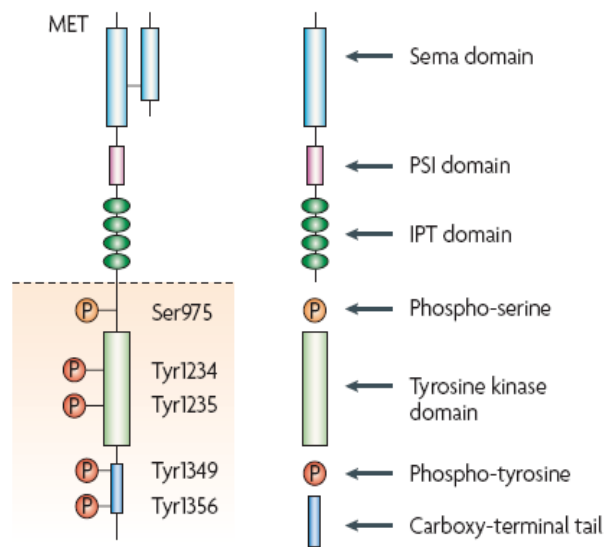
INHIBITOR	OTHER NAMES	INHIBITS
Axitinib	AG013736	VEGFR, PDGFR, and c-kit
Canertinib	CI-1033	EGFR, HER2, HER3, and HER4
Cediranib	Recentin, AZD2171	EGFR, PDGFR-β, and c-kit
Dasatinib	Sprycel, BMS-354825	Abl, Src, and Tec
Erlotinib	Tarceva, OSI-774	EGFR/HER1
Gefitinib	Iressa	EGFR/HER1
Imatinib	Gleevec, STI571	Abl, PDGFR, and c-kit
Lapatinib	Tykerb, GW-572016	EGFR and HER2
Leflunomide	Arava, SU101	PDGFR (EGFR and FGFR)
Motesanib	AMG 706	VEGFR, PDGFR, and c-kit
Neratinib	HKI-272	EGFR and HER2
Nilotinib	Tasigna	Abl, PDGFR, and c-kit
Pazopanib	Armala, GW786034	VEGFR, PDGFR-α and -β, and c-kit
Regorafenib	BAY 73-4506	VEGFR-2 and Tie-2
Semaxinib	SU5416	VEGFR
Sorafenib	Nexavar, BAY 43-9006	Raf, VEGFR-2 and -3, PDGFR-β, and c-kit
Sunitinib	Sutent, SU11248	VEGFR, PDGFR, Flt-3, c-kit, RET, and CSF-1R
Tandutinab	MLN518, CT53518	PDGFR, Flt-3, and c-kit
Vandetanib	Zactima, ZD6474	VEGFR-2, PDGFR-β, EGFR, and RET
Vatalanib	PTK787	VEGFR, PDGFR-β, and c-kit

Modified from Cancer J Clin;60:222–243 (July 2010)

Table 3: *Kinase Inhibitors*

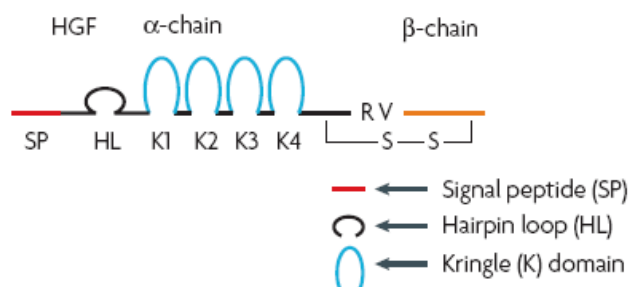## 1.2 Hepatocyte growth factor and its receptor Met

The receptor for the human hepatocyte growth factor (HGF) Met is a proto-oncogene located on chromosome 7 band 7q21–q31 and spans more than 120 kb in length. The protein is synthesized as a 170 kDa precursor which is glycosylated and proteolytically cleaved into a 50 kDa α-chain and a 140 kDa β-chain with a large extracellular region (involved in binding the ligand), a membrane spanning segment, and an intracellular region (containing the catalytic activity). The α and β chains are disulphide linked. The extracellular portion of Met contains a region of homology to semaphorins (Sema domain, which includes the full α chain and the N-terminal part of the β chain of Met), a cysteine rich Met Related Sequence (MRS) followed by glycineproline- rich (G-P) repeats, and four Immunoglobuline-like structures (Birchmeier *et al.*, 2003). The intracellular region of Met contains three regions: (1) a juxtamembrane segment that contains: (a) a serine residue (Ser 985) that, when phosphorylated by protein kinase C or by Ca2+calmodulin-dependent kinases downregulates the receptor kinase activity (Gandino *et al.*, 1994); and (b) a tyrosine (Tyr 1003) that binds the ubiquitin ligase Cbl responsible for Met polyubiquitination, endocytosis and degradation (Peschard *et al.*, 2001); (2) the tyrosine kinase domain that, upon receptor activation, undergoes transphosphorylation on Tyr1234 and Tyr1235; (3) the C-terminal region, which comprises two crucial tyrosines (Tyr1349 and Tyr1356) inserted in a degenerate motif that represents a multisubstrate docking site capable of recruiting several downstream adaptors containing Src homology-2 (SH2) domains (Figure 3).

16

Figure 3*: Structure of Met*

Hepatocyte growth factor is a member of the plasminogen related growth factor family. It is produced as inactive molecule, pro-HGF, which is subsequently cleaved to its active form by proteases such as the urokinase-type plasminogen activator and the tissue-type plasminogen activator (Maina *et al.*, 1996; Ponzetto *et al.*, 1994; Zanetti *et al.*, 1998), and by coagulation factors X, XI and XII. The mature form of HGF is a disulphide bound α–β heterodimer. It consists of a harpin loop, four Kringle domains (K1–K4) in the α-chain, and a serine protease homology domain lacking enzymatic activity in the β-chain (Figure 4).
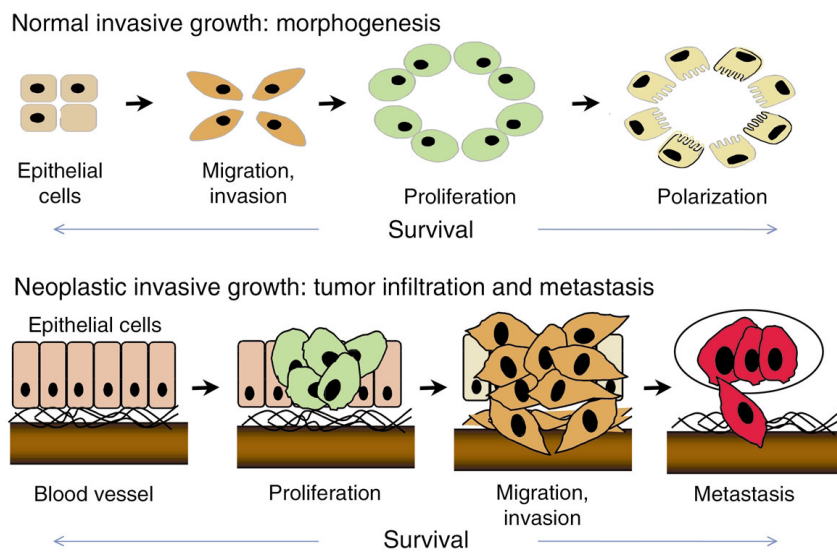
17

Figure 4: *Structure of HGF*

Met is predominantly expressed by epithelial cells (Di Renzo *et al.*, 1991; Prat *et al.*, 1991), although it is also expressed in other cell types, including endothelial (Bussolino *et al.*, 1992), myoblasts (Anastasi *et al.*, 1997), hematopoietic (Nishino *et al.*, 1995) and neuronal cells (Ebens *et al.*, 1996). HGF is expressed only by cells of mesenchymal origin (Zarnegar, 1995).

Ligand binding triggers receptor activation through dimerization and auto/trans-phosphorylation of tyrosine residues. Two tyrosine residues within the catalytic loop (Y1234, Y1235) are first to be phosphorylated, leading to full kinase activation (Longati *et al.*, 1994). Subsequently, two other tyrosine residues (Y1349, Y1356) in the carboxyl-terminal tail are phosphorylated. These are embedded in a multifunctional consensus sequence (Ponzetto *et al.*, 1994), acting as a docking site for different SH2-containing transducers and adaptors involved in several pathways, including the GRB2–SOS–RAS–RAF–MEK–ERK (extracellular signal regulated protein kinase) pathway, the phosphoinositide 3-kinase–AKT

cascade, SRC, STAT3, SHC, PLC-g, cbl, Gab1 and Rho-like GTPases such as RAC1 (Birchmeier *et al.*, 2003; Lock *et al.*, 2003). These signals direct a variety of cellular responses, such as cell proliferation, survival, motility and angiogenesis which occur during the invasive growth (Comoglio and Trusolino, 2002).

1.2.1 *Met and the invasive growth: from development to metastasis*

Invasive growth is a complex morphogenetic program characterized by independent events such as migration, survival, matrix degradation, and induction of cell polarity that occurs both during embryonic development and during adult life (Figure 5). During tissue and organ morphogenesis, normal cells colonize new territories and build polarized three-dimensional structures, thus forming the parenchymal architecture of several organs. Similarly, by the invasive growth, the angioblasts cluster and reorganize to form capillary-like structures during vasculogenesis, and new capillaries sprout from pre-existing vessels during angiogenesis (Comoglio and Trusolino, 2002).

J Clin Invest. 109(7):857-62 (Apr2002)

Figure 5: The invasive growth program under physiological and pathological conditions. In both settings, invasive growth results from analogous biological processes but the endpoints are different. Normal cells exploit invasive growth to form the parenchymal architecture of several organs. Cancer cells implement this program aberrantly to infiltrate the adjacent surroundings and form metastases.

During development, Met is required for development of the placenta, liver, kidney, neuronal, and skeletal muscle. In adults, Met expression plays a role in hematopoiesis (Ikehara, 1996; van der Voort *et al.*, 2000) including B-cells development during antigen selection (van der Voort *et al.*, 2000; van der Voort *et al.*, 1997) and tissue injury. In some instances, such as the growth and development of the liver, Met signalling induces proliferation and survival.

Under pathological conditions, invasive growth is responsible for cancer progression and metastasis (Birchmeier *et al.*, 2003; Comoglio and Trusolino, 2002). Cancer cells implement the program aberrantly to invade the adjacent compartments, overstepping the physical barriers and colonizing new territories where they may migrate, proliferate, and survive. In tumor progression Met may induce different phenotype depending on tumor stage: activating proliferation in primary tumors, stimulating motility to form micrometastases, and inducing the angiogenic phenotype to form metastases (Gao and Vande Woude, 2005).

The angiogenic properties of Met and its ligand are related to their capability to stimulate endothelial cell migration, proliferation and organization into capillary-like tubes (Bussolino *et al.*, 1992; Grant *et al.*, 1993; Silvagno *et al.*, 1995). In particular, HGF can induce pro-angiogenic cytokine expression, directly activating angiogenic pathways (Dong *et al.*, 2001; Sengupta *et al.*, 2003) or downregulating the

expression of angiogenic inhibitors (Zhang *et al.*, 2003) and promoting lymphangiogenesis (Cao *et al.*, 2006). Together, these activities favour tumor expansion, the release of neoplastic emboli into the bloodstream and Met activation in cancer cells.

The oncogenic potential of Met has been documented in a variety of human cancers and many of them appear to be dependent on receptor activity for their growth and survival. Inappropriate activation of Met can be induced by specific genetic alterations, ligand-dependent autocrine or paracrine mechanisms and receptor overexpression. Met-activating mutations are found in hereditary kidney cancer and in sporadic papillary renal cancer, childhood hepatocellular carcinoma, and gastric cancer (Lee *et al.*, 2000; Park *et al.*, 1999; Schmidt *et al.*, 1997). An autocrine or paracrine mechanism of Met activation has been reported in tumors derived from mesenchymal cells, such as osteosarcomas and rhabdomyosarcomas and from ectodermal tissues such as glioblastomas and breast carcinomas (Ferracini *et al.*, 1995; Ferracini *et al.*, 1996; Koochekpour *et al.*, 1997; Tuck *et al.*, 1996). Met overexpression is the most frequent event in human tumors and it is associated with a metastatic phenotype and a poor prognosis (Di Renzo *et al.*, 1995; Houldsworth *et al.*, 1990; Miller *et al.*, 2006; Tong *et al.*, 2004).

Since Met signalling contributes to tumor survival, growth, angiogenesis and metastasis, it provides a potential target for cancer therapy.

1.2.2. *Met receptor as a therapeutic anticancer target*

Different strategies to target Met have the aim of inhibiting tumor growth and metastatic dissemination (Comoglio *et al.*, 2008). Numerous agents have been developed and are able to target Met expression and/or
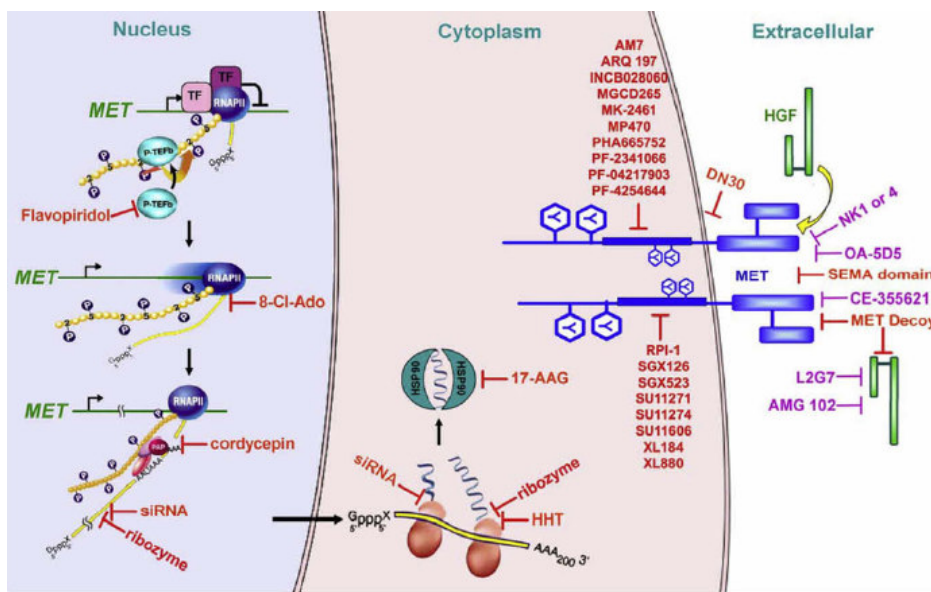
activation (Figure 6). Agents which inhibit Met expression target both ligand-dependent and -independent activation by reducing the levels of active Met to below threshold levels. The transcription inhibitors block RNAPII phosphorylation, prematurely terminate transcription and inhibit polyadenylation. Flavopiridol, a semi-synthetic flavonoid, inhibits mRNA transcription by blocking transcription elongation through the inhibition of Cdk9/ cyclin T (Chao and Price, 2001). The adenosine analog, 8-chloro-adenosine (8-Cl-Ado), is able to inhibit transcription by incorporating the triphosphate form of the analog into primarily mRNA and prematurely terminating transcription (Stellrecht *et al.*, 2003) as well as its likely inhibition on poly(A) polymerase activity (Chen and Sheppard, 2004). The polyadenylation inhibitor, cordycepin, inhibited RNA synthesis and lead to the induction of apoptosis (Chen *et al.*, 2008). Gene silencing based on antisense, ribozyme and RNA interference techniques have been also used experimentally to silence Met and HGF expression (Chu *et al.*, 2009; Corso *et al.*, 2008; Paranjpe *et al.*, 2007).

Hsp90 inhibitors such as Benzoquinone ansamycin antibiotics, including geldanamycin and its derivative 17-allylamino-17-demethoxygeldanamycin (17-AAG), inhibit Met protein stability, preventing its folding and leading to its destabilization (Webb *et al.*, 2000; Xie *et al.*, 2005).

The mature Met protein can be blocked by small molecule inhibitors, early examples for which are K252a (Morotti *et al.*, 2002), SU11274 (Berthou *et al.*, 2004) and PHA-665752 (Christensen *et al.*, 2003), all of which compete for ATP binding to block Met catalytic activity. Several of these molecules (PF-2341066, XL880, ARQ197, MK2461, MP470, SGX523 and JNJ38877605) are in phase I/II clinical trials (Comoglio *et*

al., 2008; Zou *et al.*, 2007). Owing to the way in which they were selected and the inhibitory mechanism with which they operate, none of these drugs shows absolute specificity for Met, but also target other kinases, mostly receptor tyrosine kinases (Comoglio *et al.*, 2008). Higher levels of specificity in Met inhibition can be obtained using biological molecules, either natural or designed expressly on the basis of the structure of HGF or Met. They act as antagonists that interfere in the ligand/receptor interaction, which normally leads to receptor dimerization and subsequent activation. HGF can be antagonized by its shorter forms: NK2—a natural fragment of HGF, or NK4—a truncated form of HGF, or by its uncleavable form, all of which bind the receptor but cannot fully activate it (Chan *et al.*, 1991; Matsumoto and Nakamura, 2003; Mazzone *et al.*, 2004). Similarly, anti-HGF-neutralizing antibodies act by subtracting functional HGF in the microenvironment (Cao *et al.*, 2001), some of which are in pre-clinical or phase II clinical trials (Jun *et al.*, 2007; Kim *et al.*, 2006), whereas decoy Met, a soluble truncated Met extracellular receptor domain, both sequesters the ligand and impairs receptor dimerization (Michieli *et al.*, 2004). The main limit of using HGF inhibitors is that they only inhibit HGF-dependent receptor activation. Few antagonist anti-receptor monoclonal antibodies are available; these antibodies tend to have agonist rather than antagonistic properties due to their bivalent structure (Prat *et al.*, 1998). One promising monoclonal antibody, DN-30, is able to downregulate Met and inhibit tumor growth in experimental models (Vigna *et al.*, 2008). Additional strategies to target Met signalling are aimed at Met co-receptors, such as CD44, the α6β4 integrin and B plexins (Bertotti and Comoglio, 2003). The inactivation of co-receptors could be obtained by designing antibodies or peptides

that disrupt the Met-based heterocomplexes. It has been demonstrated that a peptide fragment of CD44 prevents activation of Met-triggered signals and Met-induced migration *in vitro* (Matzke *et al.*, 2005). Finally, peptides interfering with the association of signal transducers to the Met receptor docking site could block the propagation of Met signalling. Previous studies have shown that a peptide derived from the carboxyl-terminal tail of Met specifically inhibited kinase activity and HGF-induced invasive growth in normal and transformed epithelial cells *in vitro* (Bardelli *et al.*, 1999) and enhanced the chemosensitivity of glioma cell lines to a cisplatinum derivate (Lou *et al.*, 2009).

Modified from Cancer letters;280:1-14 (2008)

Figure 6: MET targeting agents

2. Aim of the Project

The purpose of this project has been the identification of new agents able to modulate pathological neo-angiogenesis through selective pathways, overcoming or delaying the phenomena of resistance and toxicities observed during the anti-angiogenic treatment. Due to the emerging role of Met receptor and its specific ligand HGF in many aspects of cancer development and progression, Met could be considered a strong candidate for targeted therapy.

We decided to develop angiogenesis inhibitors that target the Met invasion pathway. The therapeutic inhibition of Met activity to prevent the potential dissemination of cancer cells targeting angiogenesis, suggests the use of Met inhibitors as a combination therapy that concomitantly impairs metastatic spreading and tumor neo-vascularization.

Accordingly to this, the research project has been mainly focused on the following objectives:

i. to design synthetic Met-derived peptides mimicking the docking site of the receptor;

ii. to characterize at cellular and molecular level the mechanisms of action of the peptides;

iii. to evaluate *in vitro* and *in vivo* their anti-angiogenic activity;

iv. to compare their inhibitory activity with other agents able to inactivating Met.

Experimental Design:

Peptides were delivered into cells by fusion with the internalization sequences from either the Antennapedia homeodomain (Antp) or HIV-Tat previously shown to translocate peptides efficiently across the plasma membrane (Deshayes *et al.*, 2005; Foged and Nielsen, 2008; Gupta *et al.*, 2005; Morris *et al.*, 2008; Stewart *et al.*, 2008).

The plasma membrane of eukaryotic cells constitutes an impermeable barrier for many molecules, thus, molecular transporters have been developed to facilitate the passage of pharmaceutically-active agents into living cells. Among them, much attention has recently been given to the use of peptide-based delivery systems (Deshayes *et al.*, 2005). These peptides, called cell-penetrating peptides (CPPs), are short peptides (fewer than 30 residues) with a net positive charge and acting in a receptor- and energy-independent manner. Compared with other delivery systems, such as cationic lipids (Kawakami *et al.*, 2004; Zelphati and Szoka, 1996) or polyethyleneimine (PEI) (Zaric *et al.*, 2004; Zou *et al.*, 2000), the use of peptides with cell-penetrating properties has several advantages; there is a rapid delivery of cargoes into cells with very high efficiency, stability in physiological buffers, lack of toxicity and sensitivity to serum. Moreover, CPPs avoid the endosomal pathway and favour the escape of the cargo from early endosomes (Morris *et al.*, 2008).

Many CPPs were designed from sequences of membrane interacting proteins, such as fusion proteins, signal peptides and transmembrane domains. Within these sequences, short sequences called protein transduction domains (PTDs) proved to efficiently cross biological membranes and to deliver peptides or proteins into intracellular compartments (Schwarze and Dowdy, 2000). Among these, the third

26

helix of the homeodomain of Antennapedia called Penetratin (Derossi *et al.*, 1994) and the Tat peptide derived from the transactivating protein Tat of human immunodeficiency virus-type 1 (HIV-1) (Fawell *et al.*, 1994), were used to improve the cellular uptake of peptides, proteins and oligonucleotides.

In our study we used alternatively both Penetratin and Tat sequences fused to Met-derived peptides for their delivery into endothelial cells.

3. Material and Methods

3.1. Reagents

Murine recombinant VEGF-A, human recombinant VEGFA165, murine recombinant tumor necrosis factor-α (TNF-α) and human recombinant HGF in its biologically active form (Gherardi *et al.*, 2006) were purchased from Peprotech (Offenbach, Germany); heparin was obtained from Sigma (Sigma-Aldrich Chemie, Taufkirchen, Germany). Matrigel was prepared from the Engelbreth–Holm–Swarm sarcoma as described previously (Kleinman *et al.*, 1986).

Cell-permeable peptides containing the Met docking site sequence (ref. seq: NP_000236), or a control scrambled sequence, fused to the internalization sequences of either the homeodomain 3 of the Antennapedia protein (ref. seq: NP_996167) or the Tat transactivator domain (ref. seq: NP_057853) at their N-terminus were synthesized and purified by Primm (Milan, Italy). Two Tat sequences of different lengths were used. Peptides conjugated to FAM (5-(and-6)-carboxyfluorescein) at their N-terminus were used to evaluate internalization. Peptide sequences are reported in Table 4.

Antp-Met	RQIKIWFQNRRMKWKK IGEHYVHVNATYVNVKCVA
Tat-Met	GRKKRRQRRRPP IGEHYVHVNATYVNVKCVA
Tat s-Met	RKKRRQRRR IGEHYVHVNATYVNVKCVA
Antp-scrambled	RQIKIWFQNRRMKWKKIVAVNCGVHYEHTNVKVYA
Met	IGEHYVHVNATYVNVKCVA

Table 4: *Sequences of cell-permeable peptides derived from the Met receptor docking site fused to internalization sequences from the Antennapedia and Tat proteins. Met sequence alone was used as control of cell internalization.*

3.2. Cell cultures

Human umbilical vein endothelial cells were purchased from Promo Cell (Heidelberg, Germany) and grown on 1% gelatine-coated tissue culture plates in M199 endothelial growth medium (Sigma, St Louis, MO, USA), supplemented with 10% heat-inactivated FBS (fetal bovine serum), 1% L-glutamine, fibroblast growth factors (1 mg acid-fibroblast growth factor plus 1 mg basic-fibroblast growth factor /100 ml), epidermal growth factor (1 mg/100 ml), heparin (10 mg/100 ml) and hydrocortisone (0.1 mg/100 ml). Cells were used between the second and eighth passage *in vitro*. The murine lung endothelial cell line (1G11) was the kind gift from Annunciata Vecchi (Istituto Clinico Humanitas, IRCCS and University of Milan, Milan, Italy).

3.3. Cytofluorimetric analysis

Human umbilical vein endothelial cells (6x105) were incubated with 10 µM concentrations of FAM-labeled peptides for different periods of time (10, 30, 60 and 120 min). Cells were then recovered, washed twice with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde and analyzed by flow fluorocytometry in a FACSCanto (BD Biosciences, San Jose, CA, USA).

To compare the effect of our peptides with that of the Met specific inhibitor PHA-665752 (the kind gift of Professor Livio Trusolino, Univ. Torino), $1x10^5$ HUVECs were plated in six-well plates and allowed to adhere overnight. The next day, the cells were treated in the growth medium (in the presence of HGF 50 ng/ml or 10% FBS) with the Antp-Met peptide (10 µM), Tat-Met peptide (10 µM) or Tat s-Met peptide (10 µM) or with increasing concentrations of PHA-665752 (250 and 500

29

nM, and 1 and 5 µM). Dimethyl sulfoxide and growth medium alone (in the presence of HGF 50 ng/ml or 10% FBS) were used as controls. After 2 h, cells were recovered, washed with PBS and transferred to test tubes. Cells were pelleted and resuspended in Annexin V-binding buffer (0.01M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (pH 7.4); 0.14M NaCl; 2.5mM CaCl2). Fluorescein isothiocyanate Annexin V and 7-amino-actinomycin D (BD Biosciences) were added to each test tube and incubated for 15 min at room temperature in the dark. Cells were then washed in PBS, supernatants discarded and resuspended in 400 ml of binding buffer. Samples were acquired by flow fluorocytometry using a FACSCanto (BD Biosciences) and analyzed using FACSDiva Software 6.1.2. The experiment was performed three times and each condition was a triplicate.

3.4.   *Immunofluorescence analysis*

HUVE cells were seeded at subconfluency in gelatine 0.1%-coated 8-well chamber slides in complete medium and then treated with 10 µM of each labelled-peptide. After 2 hours, cells were washed twice with PBS and fixed with 4% paraformaldehyde. After excitation at 488 nm, green fluorescent images of the internalised peptides were visualized by confocal microscopy (Leica TCS SP, Leica Lasertechnik, Heidelberg, Germany).

3.5.   *In vitro cell viability*

Human umbilical vein endothelial cell viability was evaluated in the MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. MTT is a substrate of mitochondrial reductase enzyme that is reduced to purple formazan in living cells. The absorbance of the

colored solution can be quantified by a spectrophotometer and is directly consistent with the mitochondrial activity.

Cells (1000/well) were seeded in gelatine 0.1%-coated 96-well plates in 100 µL complete medium. After complete adhesion, the medium was replaced with 2% FBS fresh medium with or without 50 ng/ml HGF and/or different peptides at different concentrations (1–50 µM) or with or without PHA-665752 at increasing concentrations (250 nM–10 µM). After the indicated times of incubation (24, 48, 72 and 96 h) 10µl/well of MTT (5mg/ml) were added to cells and after 3 hours, 100 µl of solubilization solution (3.7% HCl, 10% SDS) were used to dissolve formazan products. The plates were then read with a spectrophotometer at 570 nm wavelength.

3.6. *Migration assay*

Cell migration assays were performed in modified Boyden chambers as previously described (Albini and Benelli, 2007; Albini *et al.*, 1987). Endothelial cells ($5x10^4$) were washed with PBS, resuspended in serum-free medium, and placed in the upper compartment. The two compartments of the Boyden chamber were separated by 12 µm pore-size polycarbonate filters coated with 50 µg/ml of collagen. Fifty ng/ml HGF or 10 ng/ml VEGF diluted in serum-free M199 medium were used as chemoattractants in the lower chamber compartment and the different peptides or PHA-665752 at different concentrations were added to the cells. After 6 hours of incubation at 37°C in 5% CO_2, filters were recovered and cells on the upper surface were mechanically removed. Cells migrated to the lower filter surface were fixed with 99,8% ethanol, stained with toluidine blue and counted in double-blind manner in five to

eight consecutive fields for each sample under a microscope. Each test was performed in triplicate and repeated three times.

3.7. Invasion assay

Endothelial cells were washed with PBS, resuspended in serum-free medium, and subjected to cell invasion assays using Boyden chambers as previously described (Albini and Benelli, 2007; Albini *et al.*, 1987). Fifty ng/ml HGF diluted in serum-free M199 medium was used as chemoattractant. Invasion was performed using 12 µm pore-size polycarbonate filters pre-coated with 1mg/ml matrigel. Cells (5×10^4) were seeded in the upper compartment with or without the different peptides. At the end of the overnight incubation, cells remaining at the upper surface of the filter were mechanically removed. Cells migrated to the lower filter surface were fixed with 99,8% ethanol, stained with toluidine blue and counted in double-blind manner in five to eight consecutive fields for each sample under a microscope. Each test was performed in triplicate and repeated three times.

3.8. Morphogenesis assay

The assay was performed as described by (Grant *et al.*, 1989) A prechilled 24-well plate was carefully filled with liquid matrigel (10 mg/ml; 300µl/well) at 4°C, which was then polymerized for 1 hour at 37°. Cells (50,000/well) were suspended in serum free medium in presence of 50 ng/ml HGF or 10% FBS and with the peptides or PHA-665752 in increasing concentrations; this suspension was carefully layered on the polymerized matrigel. After 6 h incubation, the three dimensional organization of the cells was examined under an inverted

microscope (Zeiss, Oberkochen, Germany) equipped with CCD optics and a digital analysis system.

3.9. *Western blotting*

Human umbilical vein endothelial cells, pre-incubated in serum-free medium for 24 h, were pre-treated for 2 h with 10 μM Antp-Met peptide before stimulation with 10 ng/ml HGF for 15 min (Prat *et al.*, 1998). Lysates were prepared using Cell Lysis Buffer (Cell Signaling Technology, Beverly, MA, USA) and protein concentrations evaluated by the DC Protein Assay (Bio-Rad, Hercules, CA, USA). Proteins were separated on 8% SDS–polyacrylamide gel electrophoresis under reducing conditions and transferred onto polyvinylidene fluoride membranes (Amersham, Biosciences, Otelfingen, CH, USA), which were then incubated with the antibodies directed against the following human antigens: phospho-ERK1/2, tubulin, phospho-Met (tyrosine 1349), phospho-Met (tyrosines 1234–1235) (Cell Signaling Technology) and Met (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) diluted in 5% bovine serum albumin–Tris-buffered saline–0.1% Tween. Horseradish-peroxidase-conjugated secondary antibodies against rabbit or anti-mouse immunoglobulin (Cell Signaling Technology; 1:5000) were then used and reactions were visualized with the enhanced chemiluminescence kit (ECL) from Amersham Biosciences (Pittsburg, PA, USA). Lysates HUVEC and 1G11 cells were incubated with antibodies directed against human Met and mouse Met (both provided by Santa Cruz Biotechnology Inc), respectively.

33

3.10. *In vivo matrigel sponge assay*

The assay was performed as described (Albini *et al.*, 1994). HGF (50 ng/ml in the presence of heparin 50 U/ml), with or without peptides, was added to unpolymerized liquid matrigel and slowly injected subcutaneously into the flanks of 6- to 8-week-old C57/BL6 male mice (Charles River Laboratories, Calco (Lecco), Italy) in groups of 4–8 mice for each treatment. Four days after injection, the animals were killed and the pellets were removed, weighed, and either formalin-fixed and paraffin-embedded for histological examination, or minced and diluted in water for hemoglobin content measurement with a Drabkin reagent kit (Sigma). The final haemoglobin concentration was calculated from a standard calibration curve, after spectrophotometric analysis at 540 nm.

3.11. *In vivo xenograft tumor growth*

Xenografted tumors were obtained by subcutaneous injection of 5×10^6 human KS-Imm cells/mouse, and mixed with liquid matrigel into the flanks of seven-week-old male nude nu/nu (CD-1) BR mice (Charles River Laboratories) as described previously (Albini *et al.*, 2001; Albini *et al.*, 1997). Tat s-Met peptide was injected with cells (300 µl final volume of 10 µM peptide corresponding to 34 mg/ml) and then every second day by peri-tumor injection, whereas control animals received vehicle alone. In another experiment, the Antp-Met peptide (300 µl final volume of 10 µM peptide corresponding to 43 mg/ml) was injected after the tumors had become established. Animals were weighed and tumor growth monitored at regular intervals by measuring the two tumor diameters with a caliper. After the animals were killed, tumors were removed, weighed and processed for histological examination. All procedures were performed in adherence with the ECC Directive for

Care and Italian Laws on animal experimentation (Law by Decree 116/92).

3.12. *Histological analysis*

Sections of 3 µM thickness were stained with hematoxylin– eosin for histological examination. For immunohistochemistry, slides were deparaffinized in xylene, rehydrated and antigens retrieved in EDTA (pH 8); thereafter, the sections were treated with 3% of hydrogen peroxide, incubated with rabbit polyclonal CD11b/c antibody (1:600) (ABR, Golden, CO, USA) and peroxidase-coupled anti-rabbit immunoglobulin secondary antibodies, followed by reaction with 3,30-diaminobenzidine (Dako REAL EnVision system, Peroxidase/diaminobenzidineþ, Dako, Glostrup, Denmark) and finally counterstained with Mayer's hematoxylin (Sigma). For immunofluorescence microscopy, primary antibodies against von-Willibrand factor (rabbit anti-mouse, 1:100) (Millipore, Temecula, CA, USA) and Met (mouse anti-mouse, 1:100) (Santa Cruz Biotechnology Inc) were diluted in background reducing diluent (Dako) and applied for 1 h at room temperature, after the incubation with blocking serum (3% goat serum). Fluorescent secondary antibodies Alexa Fluor goat anti-rabbit 488 (1:50) (Invitrogen, Eugene, OR, USA) and goat anti-mouse 555 (1:30) (Invitrogen) were diluted in PBS buffer and applied for 1 h at room temperature. Nuclei were labeled with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA).

3.13. *Statistical analyses*

Statistical analyses were performed using two-tailed t-tests for comparison of two data sets, one-way analysis of variance for multiple data sets and two-way analysis of variance for growth curves, using the Graph Pad Prism statistics and graphing program.

4. Results

4.1. Cell internalization of the Met docking site peptide

The plasma membrane, which limits cell internalization of most proteins and peptides, can be crossed by cell-penetrating peptides, such as Tat and Antp, which can be used to deliver peptides into living cells. We verified efficient peptide internalization by incubating 5-(and-6)-carboxyfluorescein (FAM)-conjugated Tat-Met peptide (10 µM) with human umbilical vein endothelial cells (HUVEC) and monitoring fluorescence with flow cytofluorimetry. Peptide uptake was time dependent, with more than 50% of the cells being fluorescent after as little as 10 min and nearly 100% after 30 min (Figure 7).
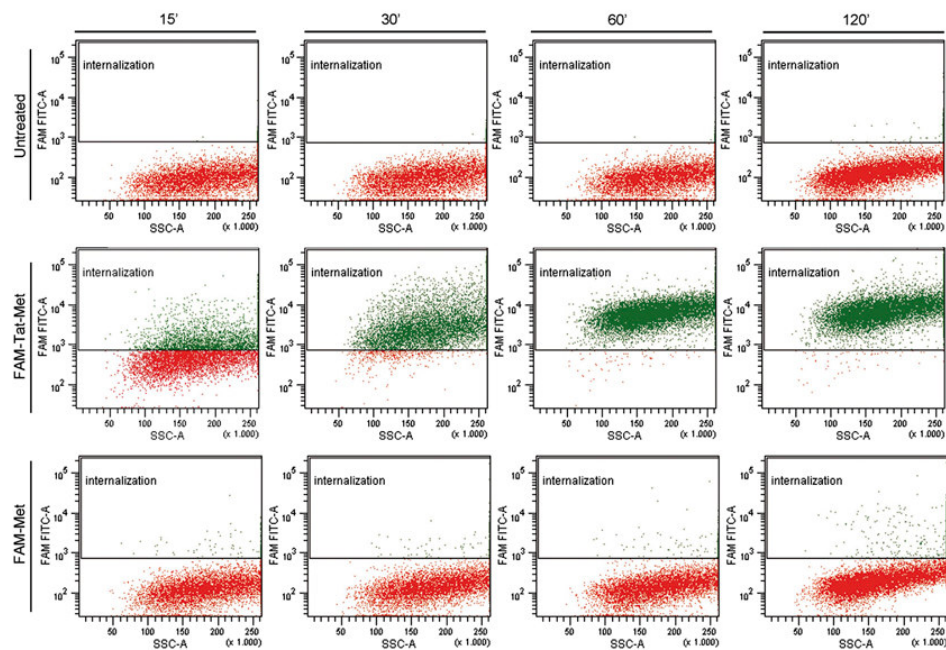

Figure 7: *The Tat-Met peptide enters into HUVECs. Tat-Met was conjugated at the N-terminus with FAM. HUVECs ($1x10^5$) were incubated for different periods of time with 10 µM peptide, washed twice and analyzed by*

fluorescence-activated cell sorting. Labelled Met peptide devoid of the cell-penetrating peptide motif and untreated cells (CTRL) were used as controls. Representative dot plots of peptide internalization percentage are shown.

A FAM-conjugated Met peptide lacking the Tat cell-penetrating peptide motif did not cross the cell membrane even after extensive incubation, showing levels of fluorescence similar to those of control untreated HUVEC.

The internalisation of labelled Tat-Met peptide was visualized even by confocal laser scanning microscopy (Figure 8).
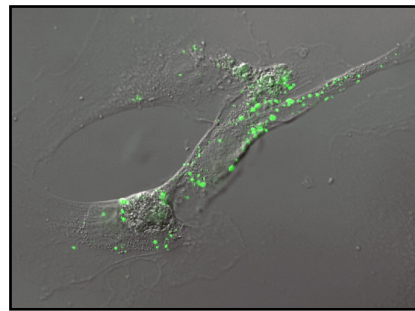

Figure 8: *Subconfluent monolayers of HUVE cells were incubated for 2 hours with 10 μM labelled Tat-Met peptide, washed and analysed by confocal microscopy. Confocal images were obtained by a Leica TCS SP confocal scan. After excitation at 488 nm, green fluorescent images of the internalised peptide were analyzed by Leica software. (Representative picture, magnification 63X).*

4.2.   Met docking site peptides inhibit HUVEC proliferation

The cell penetrating Antp-Met and Tat-Met peptides were tested for their ability to interfere with HGF-induced proliferation of HUVEC and compared with PHA-665752. Significant inhibition of cell growth in MTT assays was observed at doses of 10 μM Antp-Met already after 24 hours of treatment (Figure 9A). Higher concentrations of the Antp-Met completely abolished cell proliferation. Tat-Met and Tat s-Met

(containing a shorter Tat peptide), used at 10 µM concentrations, showed a significant reduction of cell proliferation only after 48 h (Figure 9B, C). At higher concentrations (20 µM and 50 µM) they displayed a cytostatic activity starting from 24 hours of incubation. Lower concentrations of peptides (1 µM) did not have any effect even in longer treatments.

In contrast, we observed a marked inhibitory effect starting, from the 24 hour time point when we exposed endothelial cells to micromolar concentrations (5 µM and 10 µM) of PHA-665752 (Figure 9D). Lower PHA-665752 concentrations did not exert any significant reduction on proliferation with respect to the control.

These data show that the internalized Met peptides inhibited cell proliferation, the Antp-coupled Met peptide appears to have a stronger inhibitory activity, whereas PHA-665752 required relatively high concentrations to attain comparable effects.
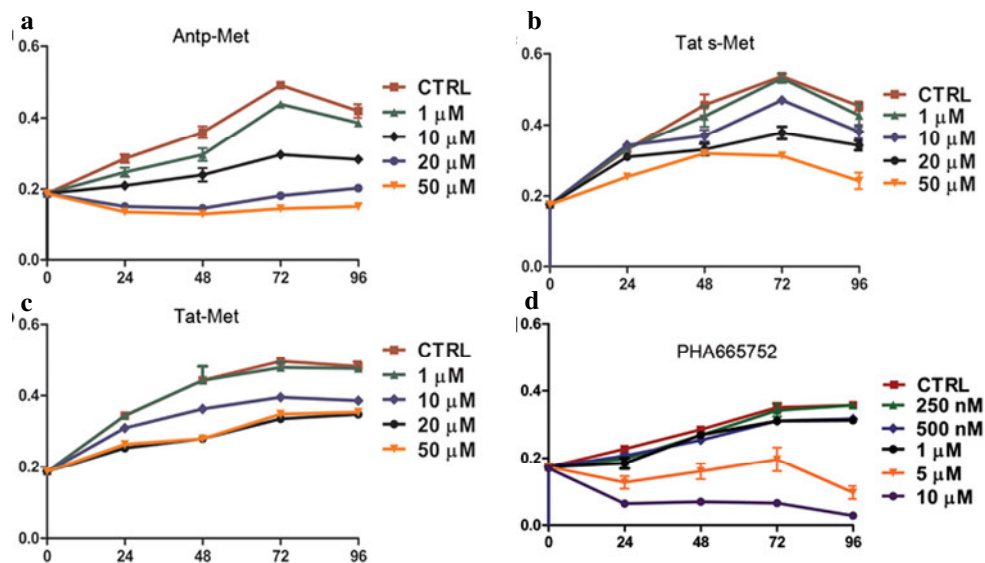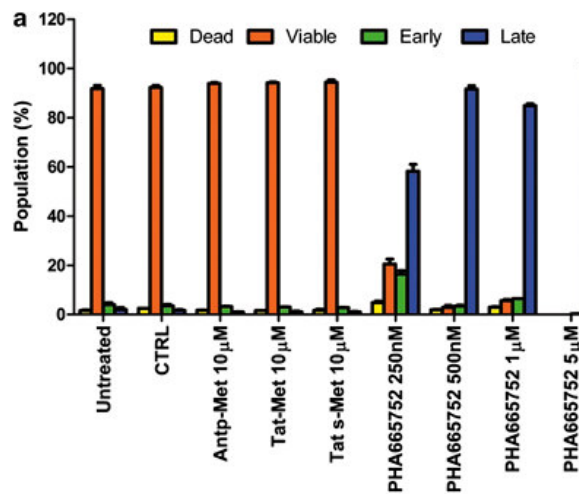
Figure 9: The Met docking site peptide inhibits HUVEC proliferation. Cell proliferation was evaluated at fixed times after the addition of HGF (50 ng/ml) ± Antp-Met (a), Tat-Met (b), Tat s-Met (c) or PHA-665752 (d) at different concentrations in 2% FBS-containing medium. The inhibitory effect of Antp-Met (10 µM) was already significant after 24 h, whereas the inhibitory effect of Tat-Met or Tat s-Met peptides were significant only after 48 h and when 20 µM concentrations were used. PHA-665752 showed an inhibitory marked reduction from 24 h at µM concentrations, whereas lower concentrations did not exert a significant reduction of proliferation with respect to the control. Means ± s.e.m. are shown (p<0.05).

4.3. Met docking site peptides do not exert toxicity or induce apoptosis in endothelial cells

The possibility that the Met inhibitors employed could exert toxicity or induce endothelial cell apoptosis, was evaluated by cytofluorimetric analysis. After only two hours of treatment of endothelial cells in the presence of HGF with 250 nM PHA-665752, approximately 60% of the cells (Figure 10A) were already undergoing apoptosis (both annexin V and 7AAD positive).
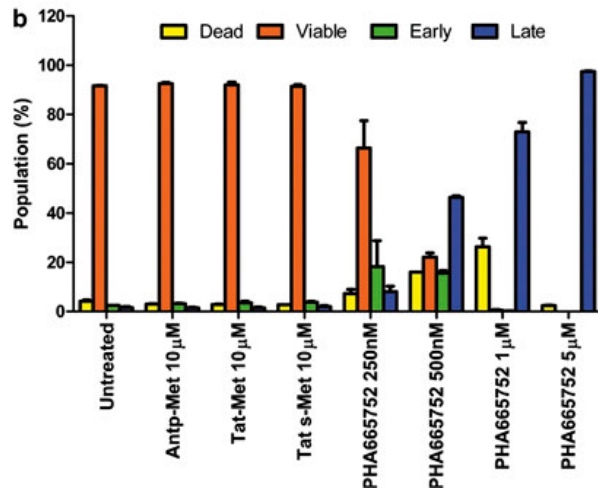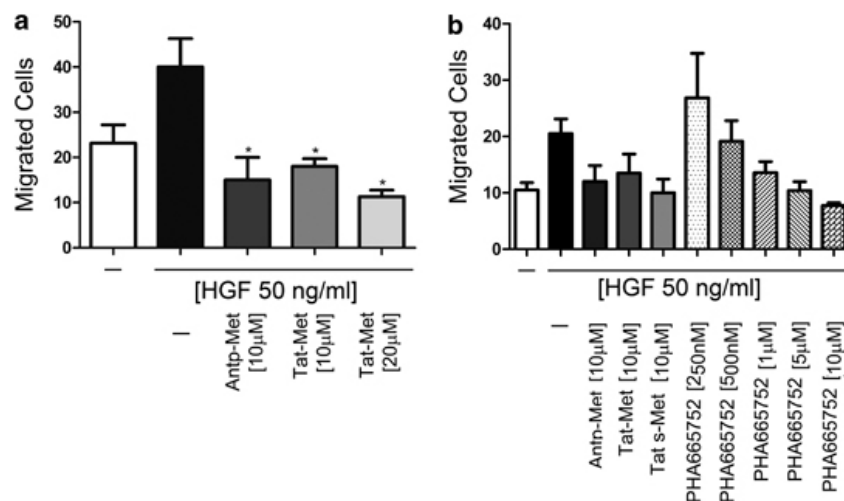
Figure 10: Met docking site peptides do not induce toxicity or apoptosis in endothelial cells. In the apoptosis assay, HUVECs were plated onto six-well plates and were treated with eithethe Antp-Met peptide (10 μM), Tat-Met peptide (10 μM) or Tat s-Met peptide (10 μM) or with increasing concentrations of PHA-665752 (250 and 500 nM, and 1 and 5 μM). Vehicle (dimethyl sulfoxide) and growth medium alone were used as controls, and HGF 50 ng/ml (panel a) or 10% FBS (panel b) were added to the growth medium. After 2 h, cells were recovered, washed and stained with two markers, Annexin V and 7-amino-actinomycin D, used to detect viable, dead or cells undergoing apoptosis. After only 2 h, 250 nM PHA-665752 induced late apoptosis in about 60% of endothelial cells in HGF-containing media (a); on the contrary, 10 μM peptides showed an high percentage of viable endothelial cells (about 95% of the population; a). The toxicity of PHA-665752 was marked both in the presence of HGF (a) or FBS (b). The experiment was performed three times and each condition was in triplicate.

In contrast, cells treated with the peptides even the highest concentrations showed a high percentage (about 95%) of viable endothelial cells and no apoptosis (Figure 10A). The toxicity of PHA-665752 was observed even in the presence of serum (FBS) with clear dose dependent effects (Figure 10B). These data suggest that the growth inhibition exerted by of PHA-665752 may be due to cytotoxicity and apoptosis.

4.4. *Met docking site peptide specifically inhibits HGF-dependent HUVEC migration and invasion*

Endothelial cells must cross basement membranes to form new blood vessels during angiogenesis; we investigated whether the peptides could affect HGF-dependent HUVEC motility and invasion. Under the effect of HGF (50 ng/ml), the number of the migrated cells in a Boyden chamber assay doubled, a non-cytotoxic dose of 10 µM peptides abrogated this migration (Figure 11A; $*p < 0.05$). No significant differences in migration inhibitory activity were found among the different peptides. We observed equivalent inhibitory effects with PHA-6657525 only in presence of the highest (cytotoxic) concentrations (5µ and 10 µM) (Figure 11B). The Met peptides did not inhibit VEGF-dependent cell migration (Figure 11C), thus showing clear specificity.

In an invasion assay performed in Boyden chambers with filters precoated with a reconstituted basement membrane (matrigel), treatment with Tat-Met or Tat s-Met significantly reduced the HGF-induced invasion (Figure 11D). Tat s-Met displayed a higher inhibitory activity than Tat-Met ($*p < 0.05$) and in both cases a dose-dependent effect was observed (Figure 11D).
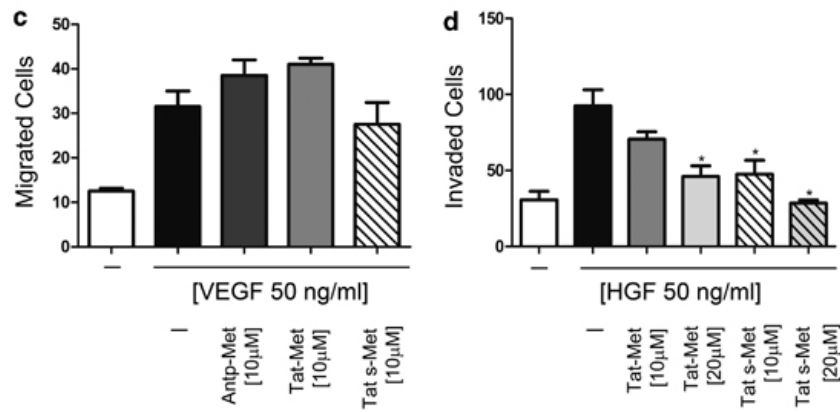

42

Figure 11: *The Met docking site peptide strongly inhibits HGF-dependent HUVEC migration and invasion. In the migration assay, HUVECs were seeded into the upper compartment of Boyden chambers in the presence or absence of Antp-, Tat- and Tat s-Met peptides or PHA-665752, whereas chemoattractants (50 ng/ml HGF (a) or 10 ng/ml VEGF (c)) were placed in the lower compartment. After 6 h incubation, cells in the upper side of the filter were removed, and cells that migrated to the lower surface were fixed, stained and counted. Serum-free medium (SFM) was used as negative control. Met mimicking cell-penetrating peptides inhibited HUVEC migration induced by HGF, but not that induced by VEGF. The inhibitor PHA-665752 showed the same inhibitory effect of peptides on HGF-dependent migration only at the highest concentrations (5 and 10 µM). The invasion assay was performed similarly, except that the filter separating the two compartments was pre-coated with matrigel and incubation was for 18 h. Tat- and Tat s-Met peptides inhibited HGF-induced matrigel invasion in dose-dependent manner (d). All experiments were performed in triplicate and repeated three times. Means ± s.e.m. are shown (*p<0.05).*

4.5. The Met docking site peptide inhibits endothelial morphogenesis in vitro

HUVECs can organize into capillary-like structures when cultured on a thick matrigel layer in presence of angiogenic factors. The Met peptides interfered with HGF-induced HUVEC morphogenesis with the same efficacy shown in inhibiting cell proliferation (Figure 12). Antp-Met was effective at 10 µM (Figure 12 E), while Tat-Met and Tat s-Met were

effective at 20 µM (Figure 12 G and I). Antp-Met did not affect FBS-dependent network formation, showing specificity of the inhibitory effect (Figure 12 K).

PHA-665752 interfered with HGF-dependent tube formation to a similar extent of the peptides only at cytotoxic micromolar concentrations (Figure 12R-S).

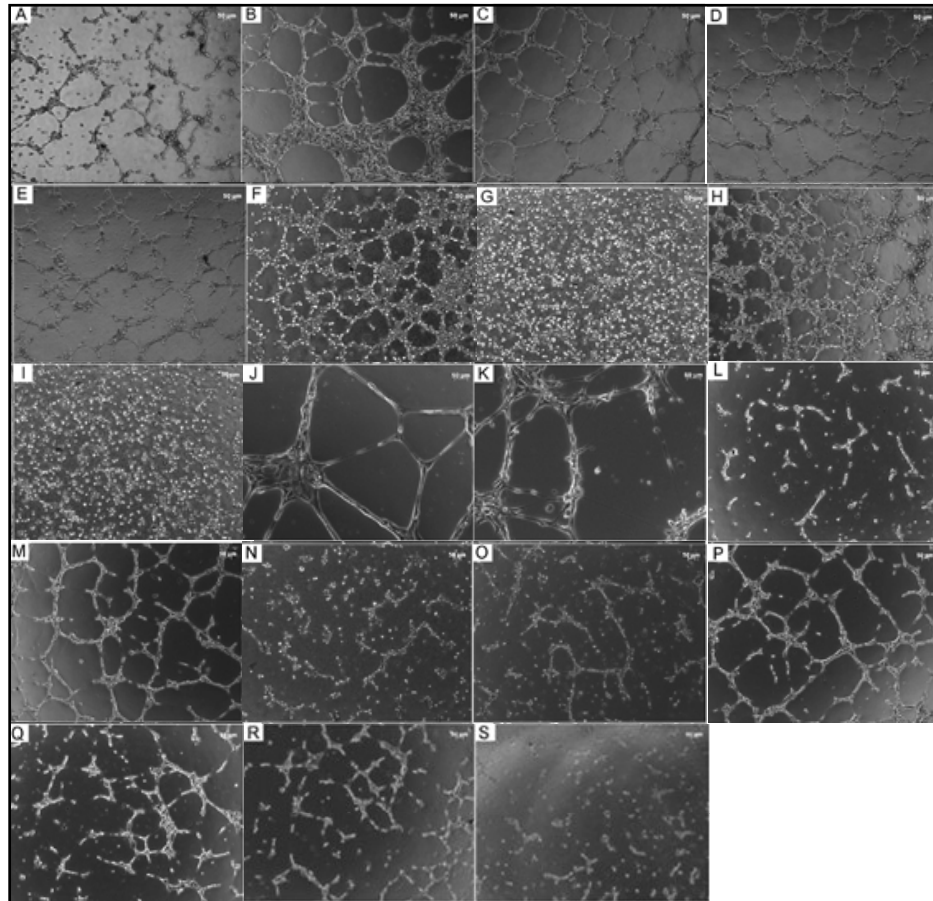***Figure 12****: The Met docking site peptide inhibits morphogenesis in vitro. HUVECs spontaneously organize in capillary-like networks after 6 h incubation at 37 °C as shown. Figure 3 C-E: effect of Antp-Met peptide on HGF-dependent tube formation. The peptide dose-dependently inhibited growth and differentiation of HUVE cells, starting at concentrations of 5 µM; inhibition was essentially complete at 10 µM, in*

the presence of HGF (magnification 5X). Figure 3 F-I: effect of Tat-Met peptides on HGF-dependent tube formation. The peptides showed an inhibitory effect at the highest concentration of 20 μM (magnification 5X). Figure 3 J-K: effect of Antp-Met peptide on FBS-dependent tube formation. The peptide did not inhibit serum-dependent growth and differentiation of HUVE cells (magnification 10X). Figure 3 L-S: effect of PHA 665752 on HGF-dependent tube formation. PHA-665752 interfered with HGF-dependent tube formation only at cytotoxic micromolar concentrations (magnification 5X).

4.6.   The Met docking site peptide inhibits HGF-dependent downstream signalling

The different biological responses triggered by HGF and mediated by Met depend on efficient downstream signal transduction, which in turn depends on receptor tyrosine phosphorylation. The binding of HGF leads to the activation of the two major signalling pathways, the ERK1/2 and the phosphoinositide 3-kinase (Birchmeier *et al.*, 2003; Boccaccio and Comoglio, 2006). We tested whether Antp-Met impaired Met receptor phosphorylation in the catalytic loop and in the docking site. While phosphorylation on tyrosines 1234-1235 localized in catalytic loop was not affected, phosphorylation on tyrosine 1349, localized in C-terminal docking site showed a 70% reduction when Antp-Met was added to cells 2 hours before stimulation with 10 ng/ml HGF (Figure 12A). Phosphorylation of ERK1/2, which is mainly involved in transducing proliferation and motility responses (Birchmeier *et al.*, 2003; Prat *et al.*, 1998), was also impaired by the Antp-Met peptide (Figure 13A). Taken together, these data show that Met-derived peptides inhibit the phosphorylation of the tyrosines located in this motif, and block activation of the downstream signalling, but do not affect phosphorylation of the tyrosines located in the activation loop.
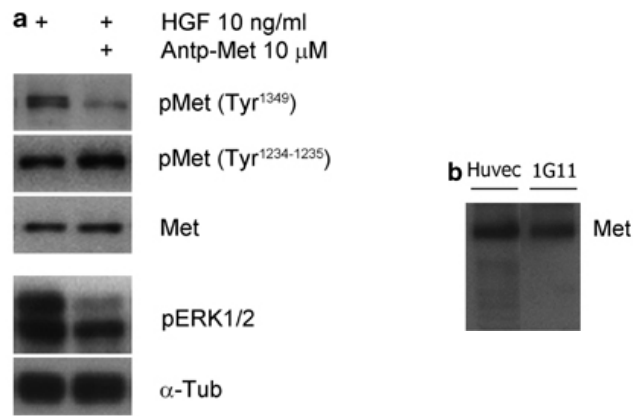
Figure 13: *The Met docking site peptide inhibits the HGF-dependent downstream signalling. Quiescent HUVECs were pretreated or not for 2 h with Antp-Met before stimulation with 10 ng/ ml HGF for 15 min, and then lysed and processed for Western blot analysis. Blots were probed with anti-phospho Met (tyrosine 1349), anti-phospho Met (tyrosines 1234–1235), anti-phospho ERK1/2, anti-Met and anti-a-tubulin. Antp-Met inhibited the phosphorylation of tyrosine 1349, but not of tyrosines 1234–1235. Besides, the phosphorylation of ERK1/2 was impaired by Antp-Met (a).(b) Human and murine endothelial cell lines, HUVEC and 1G11, respectively, express Met receptor protein.*

4.7. Met docking site peptide inhibits in vivo angiogenesis

We assessed the effect of the Met peptides on *in vivo* angiogenesis using a rapid and quantitative matrigel sponge assay. A cocktail of HGF (50 ng/ml) and heparin (50U/ml) promoted a hemorrhagic vascularization of the matrigel sponge clearly detectable at 4 days post-implantation (Figure 14A). Addition of the different Met derived peptides (10 μM) significantly inhibited the HGF-induced angiogenic response, as detected by visual inspection of the pellets and measured in a hemoglobin quantification assay (***p<0.001) (Figure 14B). The inhibition was specific, as shown by the lack of effect on the angiogenic response of an Antp-Met peptide whose sequence had been scrambled (Figure 14B). Hematoxylin-eosin stained sections of pellets showed that

46

the extensive vascularized areas in mice treated with HGF and heparin were substantially reduced in mice treated with the Met peptides (10μM) (Figure 14C (a, b, c)).

Immunohistochemistry showed that CD11b/c positive cells were readily detected within pellets containing HGF and heparin, indicating the presence of an inflammatory response; this infiltrate was significantly reduced when Met peptides were co-injected in matrigel (Figure 14C (a', b', c')).
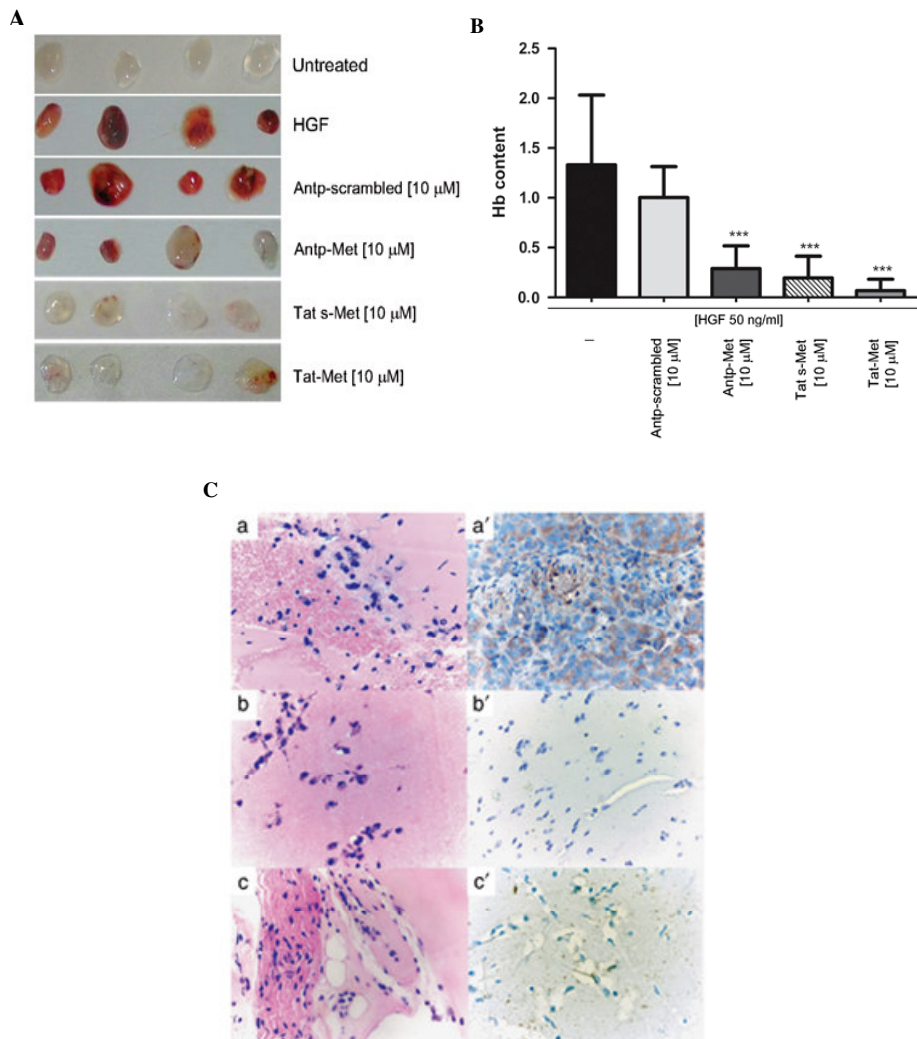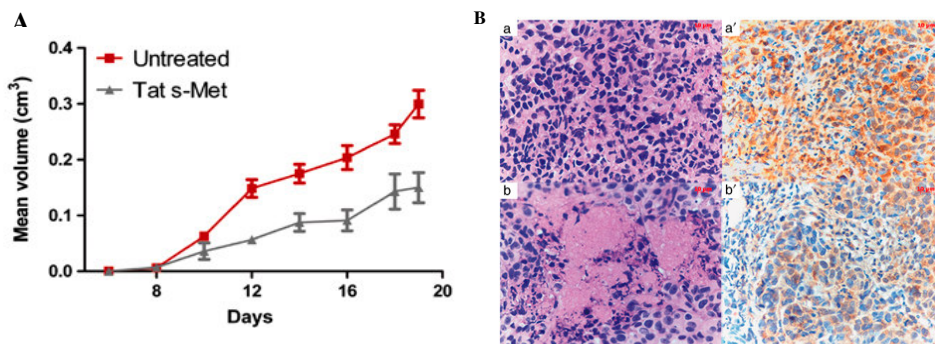
Figure 14: The Met docking site peptide inhibits in vivo angiogenesis. A cocktail of HGF (50 ng/ml) and heparin (50 U/ml) promoted a hemorrhagic vascularization of the matrigel sponge clearly detectable at 4 days post-implantation. HGF-induced vascularization of the subcutaneously injected matrigel implants was significantly inhibited by the addition of micromolar concentration of Met peptides, as detectable at visual inspection (A) and quantified by measuring hemoglobin (Hb) content of the pellets (B) (***p<0.001). Representative histological specimens that were stained with hematoxylin and eosin show extensive necrotic areas (C (a, b and c)).
CD11b/c immunohistochemistry on adjacent sections show abrogation/impairment of cellular infiltrates in the pellets treated with the peptides (C(a', b' and c')) (40X magnification).

4.8. Met docking site peptide inhibits the growth and vascularization of Kaposi's sarcomas in nude mice

We next examined whether the peptides were able effect growth and vascularization of tumors *in vivo*. KS-Imm Kaposi's Sarcoma cells were injected in liquid matrigel with or without Tat s-Met (10 µM), followed by treatment with peptides s.c. every second day until termination of the experiment. Treatment with the Tat s-Met peptide clearly inhibited tumor growth (Figure 15A). H&E staining of sections of tumors showed extensive areas of vascularization in control tumors, while extensive necrotic areas and strongly reduced vascularization were found in tumors of animals receiving peptides (Figure 15B (a, b)). Immunohistochemical staining indicated that in peptide treated tumors CD11b/c positive inflammatory cells were significantly reduced (Figure 15B (a', b')). To confirm the reduction of vascularization in tumors treated with peptide, we performed an immunofluorescence analysis for the endothelial marker von Willebrand (Figure 15C (b, b')) and we observed an higher number of von Willebrand positive cells in control tumors (Figure 15C (b)) than in tumors treated with Tat s-Met (Figure 15C (b')). We observed co-localization of the Met receptor and the von Willibrand endothelial marker in the tumors (Figure 15C (c, c')),

48

suggesting that murine tumor endothelial cells express Met. This was consistent with the presence of Met (Figure 13B) in the 1G11 murine lung microvascular endothelial cell line (Dong *et al.*, 1997).

In another set of experiments, Antp-Met, or a scrambled peptide (both at 10 µM), were injected in the peritumor area every second day after the tumor was established. Again, the peptide significantly inhibited tumor growth, while the scrambled peptide had no effect on tumor growth (Figure 15D). The central necrotic areas (Figure 15E (a, b)) and the strong inflammatory infiltrate (Figure 15E (a', b')) again were strongly reduced in presence of the Met peptide as compared to controls receiving either vehicle alone or scrambled peptide. These data indicate that the Met peptides, while not toxic for healthy endothelial cells, strongly and specifically inhibited tumor angiogenesis, inflammation and growth.
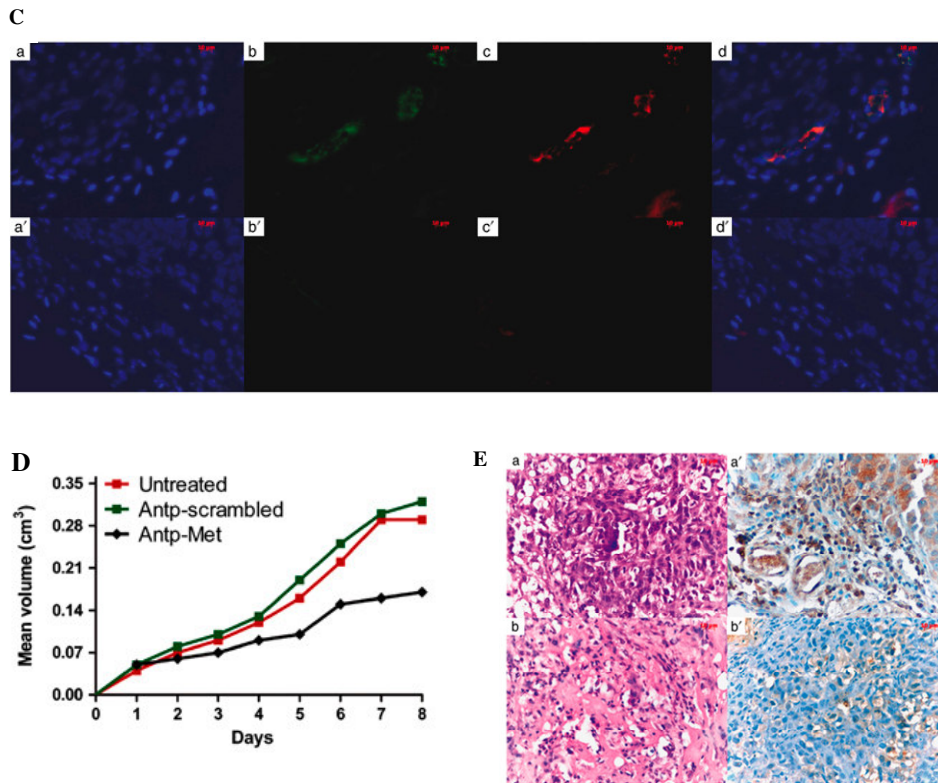
C

D

E

Figure 15: The Met docking site peptide impairs the growth and vascularization of established Kaposi's sarcomas in nude mice. Mice with xenografted Kaposi's sarcoma tumors (either established (D, E) or not (A, B)) were injected peri-tumorally with equimolar concentrations of either Antp-Met, Tat s-Met or Antp-scrambled (150 ml final volume of a 10 μM solution) or vehicle alone (control) every second day, as indicated. (A) Tumor growth analysis showed a significant inhibition of tumor growth by Tat s-Met. (B) Hematoxylin and eosin staining of the Kaposi's sarcoma xenografts grown in mice treated with Tat s-Met (b) showed extensive necrotic areas, when compared with tumors from control mice receiving only vehicle (a). Immunostaining of adjacent sections with CD11b/c, a marker of inflammatory infiltrates, showed that Tat s-Met treatment strongl inhibited the inflammation associated with the tumor (b'), relative to treatment with vehicle alone (control) (a'). (C) Immunofluorescence staining of Kaposi's sarcoma xenograft sections for the endothelial marker von Willibrand (b and b') and Met receptor (c and c'). The number of positive von Willibrand cells in control tumors (b) was much higher than that in xenografts treated with Tat-s (b'). The effect of the peptide on vascularization was Met-related, as showed by the colocalization of the Met receptor with the von Willibrand endothelial cell marker in the tumors (c and c').(D) Antp-Met peptide was also able to

50

significantly inhibit tumor growth in vivo. In contrast, a scrambled control peptide had no effect on tumor growth, showing the role of specific Met inhibition. (E) Hematoxylin and eosin staining of the Kaposi's sarcoma xenografts grown in mice treated with Antp-Met (b) showed extensive necrotic areas, as compared with tumors from control mice receiving only vehicle (a). CD11b/c immunostaining of adjacent sections also showed a strong Antp-Met inhibition of tumorassociated inflammation (b') as compared with controls (a') (40X magnification).

5.   *Discussion and conclusions*

Angiogenesis is associated with several pathologies, including chronic inflammation and arthritis, atherosclerosis, macular degeneration and cancer (Carmeliet and Jain, 2000); therapeutic strategies have envisioned angiogenesis as promising target (Carmeliet and Jain, 2000; Ferrara, 2004; Folkman, 1985; Goh *et al.*, 2007). HGF and its receptor Met are able to stimulate endothelial cell migration, proliferation and organization into capillary-like tubes and angiogenesis (Bussolino *et al.*, 1992; Grant *et al.*, 1993; Silvagno *et al.*, 1995). HGF can induce pro-angiogenic cytokine expression, directly activating angiogenic pathways (Dong *et al.*, 2001; Sengupta *et al.*, 2003) or down-regulating the expression of angiogenic inhibitors (Zhang *et al.*, 2003) and promoting lymphangiogenesis (Cao *et al.*, 2006). The HGF/Met axis has been proposed as a target for anti-angiogenic therapy (You and McDonald, 2008).

Structural and functional integrity of endothelial cells is a fundamental for vessel homeostasis and function. Alterations of endothelium are associated with increased risk for several diseases, including cardiovascular diseases and adverse events. Agents affecting endothelial integrity inducing apoptosis or direct toxicity, are likely induce vascular dysfunction. Several small molecule drugs targeting Met and other kinases show toxicity, thus biological molecules could be used to obtained higher levels of specificity and lower toxicity in Met inhibition. Here we describe the anti-angiogenic properties of synthetic cell-permeable peptides binding selectively the C-terminal tail of Met in the absence of cytotoxicity or apoptosis induction. These peptides inhibit HGF-induced proliferation, migration, invasiveness, and capillary-like

52

network formation in endothelial cells *in vitro*. Comparison of our peptides with the ATP-competitive inhibitor PHA-665752, showed that similar inhibitory effects were obtained only at high concentrations of PHA-665752 associated with apoptosis and toxicity. The ability of these peptides to interfere with pathological responses with no cytotoxic effects would be of clinical relevance to minimize the additive toxicity phenomena due to the administration of multiple inhibitor drugs.

The Met docking site peptides were active *in vivo*, blocking HGF-induced angiogenesis and restraining growth and vascularization of xenografted tumors; this is the first report of the *in vivo* inhibiting activity of a Met-specific cell penetrating peptide. Homozygous genetic deletion of either Met or HGF is lethal in utero due to defects in placental development (Bladt *et al.*, 1995; Schmidt *et al.*, 1995; Uehara *et al.*, 1995). However, the Met-/- mice did not show an endothelial phenotype (Bladt *et al.*, 1995) and the morphological appearance of endothelium in HGF-/- embryos was not altered (Uehara *et al.*, 1995). These data suggest that the Met/HGF axis may play a major role in pathological but not in physiological angiogenesis, as proposed for other angiogenic factors such as PlGF (Fischer *et al.*, 2008). This would be consistent with of Met in endothelial cells (Figure 13B), the co-expression with endothelial marker *in vivo* (Figure 15B) and the lack of toxicity *in vitro* and *in vivo*.

Biochemically, the peptides strongly impaired HGF-dependent activation of the Ras-MAPK ERK1/2 transduction pathway (Birchmeier *et al.*, 2003; Ponzetto *et al.*, 1994), consistent with previous *in vitro* studies in epithelial cells (Bardelli *et al.*, 1999). The Met-specific peptides was delivered into cells by fusion with the internalization sequences from the Antennapedia homeodomain 3 or the HIV-Tat basic

domain, which proved to act efficiently as transporters across membranes (Deshayes *et al.*, 2005; Foged and Nielsen, 2008; Gupta *et al.*, 2005; Morris *et al.*, 2008; Stewart *et al.*, 2008). A similar peptide fused to the Tat internalizing sequence enhanced *in vitro* chemosensitiviy of glioma cells to a cisplatinum derivative (Lou *et al.*, 2009). The three peptides containing the Met docking site coupled to different internalizing sequences displayed only small differences in their inhibitory efficiency *in vitro* and *in vivo*. The three peptides displayed clear specificity for Met receptor, they did not impair VEGF- or FBS-induced effects on endothelial cells *in vitro*, and a scrambled peptide was ineffective *in vivo*.

The Met-specific peptide inhibited the phosphorylation of Y1349, which binds the p85 regulatory subunit of PI3-K (Bardelli *et al.*, 1999; Graziani *et al.*, 1991; Lou *et al.*, 2009; Ponzetto *et al.*, 1994), mainly involved in transducing HGF-dependent motogenic and anti-apoptotic responses (Deregibus *et al.*, 2003; Giordano *et al.*, 2000; Khwaja *et al.*, 1998; Ponzetto *et al.*, 1993). Phosphorylation of the MAPK ERK1/2, whose activation is required for the proliferative and invasive responses (Bardelli *et al.*, 2005; Birchmeier *et al.*, 2003; Ponzetto *et al.*, 1994; Prat *et al.*, 1998), was impaired. While strongly interfering with the activation of the tyrosines located in the docking site, the peptide had no effect on phosphorylation of Y1234-1235, localized in the activation loop, as it appears to compete with substrate access to the catalytic pocket (Bardelli *et al.*, 1999).

Current clinical approaches to targeting angiogenesis have focused on blocking VEGF signalling by either anti-VEGF antibodies or multitargeted receptor tyrosine kinase inhibitors (Carmeliet and Jain, 2000; Ferrara, 2004; Folkman, 1985; Goh *et al.*, 2007; Loges *et al.*,

2009). Current clinical experience has revealed that VEGF-targeted therapy prolongs overall survival of cancer patients by only months, and experimental models have recently shown that anti-VEGF treatment inhibits primary tumor growth, but may promote invasiveness and metastasis (Ebos *et al.*, 2009; Paez-Ribes *et al.*, 2009; Pennacchietti *et al.*, 2003). In addition, the potential side effects that occur in the short-term or long-term use of the anti-VEGF treatment are becoming apparent. Some of these side effects include gastrointestinal perforations, impaired wound healing, bleeding, hypertension, proteinuria, and thrombosis (Chen and Cleck, 2009; Higa and Abraham, 2009; Zangari *et al.*, 2009). Many of the side effects are actually due to the direct effects of the drugs; cardiovascular complications are believed to be caused by the direct effects of angiogenesis inhibitors on the non tumor-associated ECs (Zangari *et al.*, 2009). It has also been demonstrated that most tumors develop mechanisms of resistance to anti-angiogenic treatment (Eikesdal and Kalluri, 2009) as a consequence of the long-term administration of anti-angiogenesis inhibitors. Because multiple signalling pathways are involved in angiogenesis, there is a signalling redundancy, and the block of a single pathway may not be highly effective and/or can lead to resistance when the tumor cells develop other angiogenesis mechanisms (Eikesdal and Kalluri, 2009).

By targeting multiple pathways, resistance may be able to be overcome or delayed, making combination drug therapies important in the design of future clinical trials. The fact that Met is both a pro-angiogenic factor and an oncogene suggested that it may be an optimal target for anti-cancer treatment, simultaneously inhibiting angiogenesis and oncogenesis (Michieli *et al.*, 2004) and potentially complementing VEGF targeted anti-angiogenic therapy.

Our observations provide the novel result that Met-specific peptides mimicking the tail multifunctional docking site can block angiogenesis and consequent cancer growth and related metastatic dissemination.

6. References

o Abraham JA, Mergia A, Whang JL, Tumolo A, Friedman J, Hjerrild KA *et al* (1986). Nucleotide sequence of a bovine clone encoding the angiogenic protein, basic fibroblast growth factor. *Science* **233:** 545-8.

o Adini A, Kornaga T, Firoozbakht F, Benjamin LE (2002). Placental growth factor is a survival factor for tumor endothelial cells and macrophages. *Cancer Res* **62:** 2749-52.

o Albini A, Benelli R (2007). The chemoinvasion assay: a method to assess tumor and endothelial cell invasion and its modulation. *Nat Protoc* **2:** 504-11.

o Albini A, Fontanini G, Masiello L, Tacchetti C, Bigini D, Luzzi P *et al* (1994). Angiogenic potential in vivo by Kaposi's sarcoma cell-free supernatants and HIV-1 tat product: inhibition of KS-like lesions by tissue inhibitor of metalloproteinase-2. *AIDS* **8:** 1237-44.

o Albini A, Iwamoto Y, Kleinman HK, Martin GR, Aaronson SA, Kozlowski JM *et al* (1987). A rapid in vitro assay for quantitating the invasive potential of tumor cells. *Cancer Res* **47:** 3239-45.

o Albini A, Morini M, D'Agostini F, Ferrari N, Campelli F, Arena G *et al* (2001). Inhibition of angiogenesis-driven Kaposi's sarcoma tumor growth in nude mice by oral N-acetylcysteine. *Cancer Res* **61:** 8171-8.

o Albini A, Paglieri I, Orengo G, Carlone S, Aluigi MG, DeMarchi R *et al* (1997). The beta-core fragment of human chorionic gonadotrophin inhibits growth of Kaposi's sarcoma-derived cells and a new immortalized Kaposi's sarcoma cell line. *AIDS* **11:** 713-21.

o Alexander JS, Elrod JW (2002). Extracellular matrix, junctional integrity and matrix metalloproteinase interactions in endothelial permeability regulation. *J Anat* **200:** 561-74.

o Anastasi S, Giordano S, Sthandier O, Gambarotta G, Maione R, Comoglio P *et al* (1997). A natural hepatocyte growth factor/scatter factor autocrine loop in myoblast cells and the effect of the constitutive

Met kinase activation on myogenic differentiation. *J Cell Biol* **137:** 1057-68.

o Andrae J, Gallini R, Betsholtz C (2008). Role of platelet-derived growth factors in physiology and medicine. *Genes Dev* **22:** 1276-312.

o Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T *et al* (1997). Isolation of putative progenitor endothelial cells for angiogenesis. *Science* **275:** 964-7.

o Bardelli A, Longati P, Williams TA, Benvenuti S, Comoglio PM (1999). A peptide representing the carboxyl-terminal tail of the met receptor inhibits kinase activity and invasive growth. *J Biol Chem* **274:** 29274-81.

o Bardelli C, Sala M, Cavallazzi U, Prat M (2005). Agonist Met antibodies define the signalling threshold required for a full mitogenic and invasive program of Kaposi's Sarcoma cells. *Biochem Biophys Res Commun* **334:** 1172-9.

o Barleon B, Siemeister G, Martiny-Baron G, Weindel K, Herzog C, Marme D (1997). Vascular endothelial growth factor up-regulates its receptor fms-like tyrosine kinase 1 (FLT-1) and a soluble variant of FLT-1 in human vascular endothelial cells. *Cancer Res* **57:** 5421-5.

o Bellik L, Vinci MC, Filippi S, Ledda F, Parenti A (2005). Intracellular pathways triggered by the selective FLT-1-agonist placental growth factor in vascular smooth muscle cells exposed to hypoxia. *Br J Pharmacol* **146:** 568-75.

o Bergers G, Benjamin LE (2003). Tumorigenesis and the angiogenic switch. *Nat Rev Cancer* **3:** 401-10.

o Berthou S, Aebersold DM, Schmidt LS, Stroka D, Heigl C, Streit B *et al* (2004). The Met kinase inhibitor SU11274 exhibits a selective inhibition pattern toward different receptor mutated variants. *Oncogene* **23:** 5387-93.

o Bertotti A, Comoglio PM (2003). Tyrosine kinase signal specificity: lessons from the HGF receptor. *Trends Biochem Sci* **28:** 527-33.

o Birchmeier C, Birchmeier W, Gherardi E, Vande Woude GF (2003). Met, metastasis, motility and more. *Nat Rev Mol Cell Biol* **4:** 915-25.

o Bladt F, Riethmacher D, Isenmann S, Aguzzi A, Birchmeier C (1995). Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. *Nature* **376:** 768-71.

o Blobe GC, Schiemann WP, Lodish HF (2000). Role of transforming growth factor beta in human disease. *N Engl J Med* **342:** 1350-8.

o Boccaccio C, Comoglio PM (2006). Invasive growth: a MET-driven genetic programme for cancer and stem cells. *Nat Rev Cancer* **6:** 637-45.

o Bussolino F, Di Renzo MF, Ziche M, Bocchietto E, Olivero M, Naldini L *et al* (1992). Hepatocyte growth factor is a potent angiogenic factor which stimulates endothelial cell motility and growth. *J Cell Biol* **119:** 629-41.

o Cantelmo AR, Cammarota R, Noonan DM, Focaccetti C, Comoglio PM, Prat M *et al* Cell delivery of Met docking site peptides inhibit angiogenesis and vascular tumor growth. *Oncogene* **29:** 5286-98.

o Cao B, Su Y, Oskarsson M, Zhao P, Kort EJ, Fisher RJ *et al* (2001). Neutralizing monoclonal antibodies to hepatocyte growth factor/scatter factor (HGF/SF) display antitumor activity in animal models. *Proc Natl Acad Sci U S A* **98:** 7443-8.

o Cao R, Bjorndahl MA, Gallego MI, Chen S, Religa P, Hansen AJ *et al* (2006). Hepatocyte growth factor is a lymphangiogenic factor with an indirect mechanism of action. *Blood* **107:** 3531-6.

o Cao Y, Cao R, Hedlund EM (2008). R Regulation of tumor angiogenesis and metastasis by FGF and PDGF signaling pathways. *J Mol Med* **86:** 785-9.

o Carmeliet P (2003). Angiogenesis in health and disease. *Nat Med* **9:** 653-60.

o Carmeliet P, Jain RK (2000). Angiogenesis in cancer and other diseases. *Nature* **407:** 249-57.

o Carmeliet P, Moons L, Luttun A, Vincenti V, Compernolle V, De Mol M *et al* (2001). Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. *Nat Med* **7:** 575-83.

o Chan AM, Rubin JS, Bottaro DP, Hirschfield DW, Chedid M, Aaronson SA (1991). Identification of a competitive HGF antagonist encoded by an alternative transcript. *Science* **254:** 1382-5.

o Chao SH, Price DH (2001). Flavopiridol inactivates P-TEFb and blocks most RNA polymerase II transcription in vivo. *J Biol Chem* **276:** 31793-9.

o Chen HX, Cleck JN (2009). Adverse effects of anticancer agents that target the VEGF pathway. *Nat Rev Clin Oncol* **6:** 465-77.

o Chen LS, Sheppard TL (2004). Chain termination and inhibition of Saccharomyces cerevisiae poly(A) polymerase by C-8-modified ATP analogs. *J Biol Chem* **279:** 40405-11.

o Chen LS, Stellrecht CM, Gandhi V (2008). RNA-directed agent, cordycepin, induces cell death in multiple myeloma cells. *Br J Haematol* **140:** 682-391.

o Christensen JG, Schreck R, Burrows J, Kuruganti P, Chan E, Le P *et al* (2003). A selective small molecule inhibitor of c-Met kinase inhibits c-Met-dependent phenotypes in vitro and exhibits cytoreductive antitumor activity in vivo. *Cancer Res* **63:** 7345-55.

o Chu SH, Feng DF, Zhang H, Chen ET, Duan ZX, Li XY *et al* (2009). c-Met-targeted RNA interference inhibits growth and metastasis of glioma U251 cells in vitro. *J Neurooncol* **93:** 183-9.

o Comoglio PM, Giordano S, Trusolino L (2008). Drug development of MET inhibitors: targeting oncogene addiction and expedience. *Nat Rev Drug Discov* **7:** 504-16.

o Comoglio PM, Trusolino L (2002). Invasive growth: from development to metastasis. *J Clin Invest* **109:** 857-62.

o Cook KM, Figg WD Angiogenesis inhibitors: current strategies and future prospects. *CA Cancer J Clin* **60:** 222-43.

o Corso S, Migliore C, Ghiso E, De Rosa G, Comoglio PM, Giordano S (2008). Silencing the MET oncogene leads to regression of experimental tumors and metastases. *Oncogene* **27:** 684-93.

o Database of Current Clinical Trials. 2009. Bethesda, MD: US National Library of Medicine; 2009. Available at: http://www.clinicaltrials.gov. Accessed September 2009

o De Palma M, Venneri MA, Roca C, Naldini L (2003). Targeting exogenous genes to tumor angiogenesis by transplantation of genetically modified hematopoietic stem cells. *Nat Med* **9:** 789-95.

o Deregibus MC, Buttiglieri S, Russo S, Bussolati B, Camussi G (2003). CD40-dependent activation of phosphatidylinositol 3-kinase/Akt pathway mediates endothelial cell survival and in vitro angiogenesis. *J Biol Chem* **278:** 18008-14.

o Derossi D, Joliot AH, Chassaing G, Prochiantz A (1994). The third helix of the Antennapedia homeodomain translocates through biological membranes. *J Biol Chem* **269:** 10444-50.

o Deshayes S, Morris MC, Divita G, Heitz F (2005). Cell-penetrating peptides: tools for intracellular delivery of therapeutics. *Cell Mol Life Sci* **62:** 1839-49.

o Di Renzo MF, Narsimhan RP, Olivero M, Bretti S, Giordano S, Medico E *et al* (1991). Expression of the Met/HGF receptor in normal and neoplastic human tissues. *Oncogene* **6:** 1997-2003.

o Di Renzo MF, Olivero M, Giacomini A, Porte H, Chastre E, Mirossay L *et al* (1995). Overexpression and amplification of the met/HGF receptor gene during the progression of colorectal cancer. *Clin Cancer Res* **1:** 147-54.

o Dong G, Chen Z, Li ZY, Yeh NT, Bancroft CC, Van Waes C (2001). Hepatocyte growth factor/scatter factor-induced activation of MEK and PI3K signal pathways contributes to expression of proangiogenic cytokines interleukin-8 and vascular endothelial growth

factor in head and neck squamous cell carcinoma. *Cancer Res* **61:** 5911-8.

o Dong QG, Bernasconi S, Lostaglio S, De Calmanovici RW, Martin-Padura I, Breviario F *et al* (1997). A general strategy for isolation of endothelial cells from murine tissues. Characterization of two endothelial cell lines from the murine lung and subcutaneous sponge implants. *Arterioscler Thromb Vasc Biol* **17:** 1599-604.

o Ebens A, Brose K, Leonardo ED, Hanson MG, Jr., Bladt F, Birchmeier C *et al* (1996). Hepatocyte growth factor/scatter factor is an axonal chemoattractant and a neurotrophic factor for spinal motor neurons. *Neuron* **17:** 1157-72.

o Ebos JM, Lee CR, Cruz-Munoz W, Bjarnason GA, Christensen JG, Kerbel RS (2009). Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. *Cancer Cell* **15:** 232-9.

o Eikesdal HP, Kalluri R (2009). Drug resistance associated with antiangiogenesis therapy. *Semin Cancer Biol* **19:** 310-7.

o Escudier B, Szczylik C, Hutson TE, Demkow T, Staehler M, Rolland F *et al* (2009). Randomized phase II trial of first-line treatment with sorafenib versus interferon Alfa-2a in patients with metastatic renal cell carcinoma. *J Clin Oncol* **27:** 1280-9.

o Eskens FA, Verweij J (2006). The clinical toxicity profile of vascular endothelial growth factor (VEGF) and vascular endothelial growth factor receptor (VEGFR) targeting angiogenesis inhibitors; a review. *Eur J Cancer* **42:** 3127-39.

o Fawell S, Seery J, Daikh Y, Moore C, Chen LL, Pepinsky B *et al* (1994). Tat-mediated delivery of heterologous proteins into cells. *Proc Natl Acad Sci U S A* **91:** 664-8.

o Ferracini R, Di Renzo MF, Scotlandi K, Baldini N, Olivero M, Lollini P *et al* (1995). The Met/HGF receptor is over-expressed in human osteosarcomas and is activated by either a paracrine or an autocrine circuit. *Oncogene* **10:** 739-49.

o Ferracini R, Olivero M, Di Renzo MF, Martano M, De Giovanni C, Nanni P *et al* (1996). Retrogenic expression of the MET proto-

oncogene correlates with the invasive phenotype of human rhabdomyosarcomas. *Oncogene* **12:** 1697-705.

o Ferrara N (2000a). Vascular endothelial growth factor and the regulation of angiogenesis. *Recent Prog Horm Res* **55:** 15-35; discussion 35-6.

o Ferrara N (2000b). VEGF: an update on biological and therapeutic aspects. *Curr Opin Biotechnol* **11:** 617-24.

o Ferrara N (2002). VEGF and the quest for tumour angiogenesis factors. *Nat Rev Cancer* **2:** 795-803.

o Ferrara N (2004). Vascular endothelial growth factor: basic science and clinical progress. *Endocr Rev* **25:** 581-611.

o Ferrara N, Gerber HP, LeCouter J (2003). The biology of VEGF and its receptors. *Nat Med* **9:** 669-76.

o Fischer C, Mazzone M, Jonckx B, Carmeliet P (2008). FLT1 and its ligands VEGFB and PlGF: drug targets for anti-angiogenic therapy? *Nat Rev Cancer* **8:** 942-56.

o Foged C, Nielsen HM (2008). Cell-penetrating peptides for drug delivery across membrane barriers. *Expert Opin Drug Deliv* **5:** 105-17.

o Folberg R, Maniotis AJ (2004). Vasculogenic mimicry. *APMIS* **112:** 508-25.

o Folkman J (1971). Tumor angiogenesis: therapeutic implications. *N Engl J Med* **285:** 1182-6.

o Folkman J (1972). Anti-angiogenesis: new concept for therapy of solid tumors. *Ann Surg* **175:** 409-16.

o Folkman J (1985). Tumor angiogenesis. *Adv Cancer Res* **43:** 175-203.

o Gan HK, Seruga B, Knox JJ (2009). Sunitinib in solid tumors. *Expert Opin Investig Drugs* **18:** 821-34.

o   Gandino L, Longati P, Medico E, Prat M, Comoglio PM (1994). Phosphorylation of serine 985 negatively regulates the hepatocyte growth factor receptor kinase. *J Biol Chem* **269:** 1815-20.

o   Gao CF, Vande Woude GF (2005). HGF/SF-Met signaling in tumor progression. *Cell Res* **15:** 49-51.

o   Gerber HP, Condorelli F, Park J, Ferrara N (1997). Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes. Flt-1, but not Flk-1/KDR, is up-regulated by hypoxia. *J Biol Chem* **272:** 23659-67.

o   Gherardi E, Sandin S, Petoukhov MV, Finch J, Youles ME, Ofverstedt LG *et al* (2006). Structural basis of hepatocyte growth factor/scatter factor and MET signalling. *Proc Natl Acad Sci U S A* **103:** 4046-51.

o   Giordano S, Maffe A, Williams TA, Artigiani S, Gual P, Bardelli A *et al* (2000). Different point mutations in the met oncogene elicit distinct biological properties. *FASEB J* **14:** 399-406.

o   Goh PP, Sze DM, Roufogalis BD (2007). Molecular and cellular regulators of cancer angiogenesis. *Curr Cancer Drug Targets* **7:** 743-58.

o   Grant DS, Kleinman HK, Goldberg ID, Bhargava MM, Nickoloff BJ, Kinsella JL *et al* (1993). Scatter factor induces blood vessel formation in vivo. *Proc Natl Acad Sci U S A* **90:** 1937-41.

o   Grant DS, Tashiro K, Segui-Real B, Yamada Y, Martin GR, Kleinman HK (1989). Two different laminin domains mediate the differentiation of human endothelial cells into capillary-like structures in vitro. *Cell* **58:** 933-43.

o   Graziani A, Gramaglia D, Cantley LC, Comoglio PM (1991). The tyrosine-phosphorylated hepatocyte growth factor/scatter factor receptor associates with phosphatidylinositol 3-kinase. *J Biol Chem* **266:** 22087-90.

o   Gupta B, Levchenko TS, Torchilin VP (2005). Intracellular delivery of large molecules and small particles by cell-penetrating proteins and peptides. *Adv Drug Deliv Rev* **57:** 637-51.

o   Hanahan D, Folkman J (1996). Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* **86:** 353-64.

o   Hattori K, Dias S, Heissig B, Hackett NR, Lyden D, Tateno M *et al* (2001). Vascular endothelial growth factor and angiopoietin-1 stimulate postnatal hematopoiesis by recruitment of vasculogenic and hematopoietic stem cells. *J Exp Med* **193:** 1005-14.

o   Heath VL, Bicknell R (2009). Anticancer strategies involving the vasculature. *Nat Rev Clin Oncol* **6:** 395-404.

o   Higa GM, Abraham J (2009). Biological mechanisms of bevacizumab-associated adverse events. *Expert Rev Anticancer Ther* **9:** 999-1007.

o   Hood JD, Meininger CJ, Ziche M, Granger HJ (1998). VEGF upregulates ecNOS message, protein, and NO production in human endothelial cells. *Am J Physiol* **274:** H1054-8.

o   Houldsworth J, Cordon-Cardo C, Ladanyi M, Kelsen DP, Chaganti RS (1990). Gene amplification in gastric and esophageal adenocarcinomas. *Cancer Res* **50:** 6417-22.

o   Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W *et al* (2004). Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N Engl J Med* **350:** 2335-42.

o   Ikehara S (1996). Role of hepatocyte growth factor in hemopoiesis. *Leuk Lymphoma* **23:** 297-303.

o   Jain RK (1999). Transport of molecules, particles, and cells in solid tumors. *Annu Rev Biomed Eng* **1:** 241-63.

o   Johnson DH, Fehrenbacher L, Novotny WF, Herbst RS, Nemunaitis JJ, Jablons DM *et al* (2004). Randomized phase II trial comparing bevacizumab plus carboplatin and paclitaxel with carboplatin and paclitaxel alone in previously untreated locally advanced or metastatic non-small-cell lung cancer. *J Clin Oncol* **22:** 2184-91.

o Jun HT, Sun J, Rex K, Radinsky R, Kendall R, Coxon A *et al* (2007). AMG 102, a fully human anti-hepatocyte growth factor/scatter factor neutralizing antibody, enhances the efficacy of temozolomide or docetaxel in U-87 MG cells and xenografts. *Clin Cancer Res* **13:** 6735-42.

o Kawakami S, Harada A, Sakanaka K, Nishida K, Nakamura J, Sakaeda T *et al* (2004). In vivo gene transfection via intravitreal injection of cationic liposome/plasmid DNA complexes in rabbits. *Int J Pharm* **278:** 255-62.

o Kerbel RS (2008). Tumor angiogenesis. *N Engl J Med* **358:** 2039-49.

o Khwaja A, Lehmann K, Marte BM, Downward J (1998). Phosphoinositide 3-kinase induces scattering and tubulogenesis in epithelial cells through a novel pathway. *J Biol Chem* **273:** 18793-801.

o Kim KJ, Wang L, Su YC, Gillespie GY, Salhotra A, Lal B *et al* (2006). Systemic anti-hepatocyte growth factor monoclonal antibody therapy induces the regression of intracranial glioma xenografts. *Clin Cancer Res* **12:** 1292-8.

o Klagsbrun M (1991). Regulators of angiogenesis: stimulators, inhibitors, and extracellular matrix. *J Cell Biochem* **47:** 199-200.

o Kleinman HK, McGarvey ML, Hassell JR, Star VL, Cannon FB, Laurie GW *et al* (1986). Basement membrane complexes with biological activity. *Biochemistry* **25:** 312-8.

o Koochekpour S, Jeffers M, Rulong S, Taylor G, Klineberg E, Hudson EA *et al* (1997). Met and hepatocyte growth factor/scatter factor expression in human gliomas. *Cancer Res* **57:** 5391-8.

o Lee JH, Han SU, Cho H, Jennings B, Gerrard B, Dean M *et al* (2000). A novel germ line juxtamembrane Met mutation in human gastric cancer. *Oncogene* **19:** 4947-53.

o Lindahl P, Johansson BR, Leveen P, Betsholtz C (1997). Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. *Science* **277:** 242-5.

o   Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF *et al* (2008). Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* **359:** 378-90.

o   Lock LS, Frigault MM, Saucier C, Park M (2003). Grb2-independent recruitment of Gab1 requires the C-terminal lobe and structural integrity of the Met receptor kinase domain. *J Biol Chem* **278:** 30083-90.

o   Loges S, Mazzone M, Hohensinner P, Carmeliet P (2009). Silencing or fueling metastasis with VEGF inhibitors: antiangiogenesis revisited. *Cancer Cell* **15:** 167-70.

o   Longati P, Bardelli A, Ponzetto C, Naldini L, Comoglio PM (1994). Tyrosines1234-1235 are critical for activation of the tyrosine kinase encoded by the MET proto-oncogene (HGF receptor). *Oncogene* **9:** 49-57.

o   Lou X, Zhou Q, Yin Y, Zhou C, Shen Y (2009). Inhibition of the met receptor tyrosine kinase signaling enhances the chemosensitivity of glioma cell lines to CDDP through activation of p38 MAPK pathway. *Mol Cancer Ther*.

o   Maciag T, Mehlman T, Friesel R, Schreiber AB (1984). Heparin binds endothelial cell growth factor, the principal endothelial cell mitogen in bovine brain. *Science* **225:** 932-5.

o   Maglione D, Guerriero V, Viglietto G, Delli-Bovi P, Persico MG (1991). Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor. *Proc Natl Acad Sci U S A* **88:** 9267-71.

o   Mahon FX, Rea D, Guilhot J, Guilhot F, Huguet F, Nicolini F *et al* Discontinuation of imatinib in patients with chronic myeloid leukaemia who have maintained complete molecular remission for at least 2 years: the prospective, multicentre Stop Imatinib (STIM) trial. *Lancet Oncol* **11:** 1029-1035.

o   Maina F, Casagranda F, Audero E, Simeone A, Comoglio PM, Klein R *et al* (1996). Uncoupling of Grb2 from the Met receptor in vivo reveals complex roles in muscle development. *Cell* **87:** 531-42.

o Masuda M, Yasuhara S, Yamashita M, Shibuya M, Odaka T (1990). Nucleotide sequence of the murine thyroid hormone receptor (alpha-1) cDNA. *Nucleic Acids Res* **18:** 3055.

o Matsumoto K, Nakamura T (2003). NK4 (HGF-antagonist/angiogenesis inhibitor) in cancer biology and therapeutics. *Cancer Sci* **94:** 321-7.

o Matsumoto T, Claesson-Welsh L (2001). VEGF receptor signal transduction. *Sci STKE* **2001:** re21.

o Matzke A, Herrlich P, Ponta H, Orian-Rousseau V (2005). A five-amino-acid peptide blocks Met- and Ron-dependent cell migration. *Cancer Res* **65:** 6105-10.

o Mazzone M, Basilico C, Cavassa S, Pennacchietti S, Risio M, Naldini L *et al* (2004). An uncleavable form of pro-scatter factor suppresses tumor growth and dissemination in mice. *J Clin Invest* **114:** 1418-32.

o McDonald DM, Baluk P (2002). Significance of blood vessel leakiness in cancer. *Cancer Res* **62:** 5381-5.

o Meyer M, Clauss M, Lepple-Wienhues A, Waltenberger J, Augustin HG, Ziche M *et al* (1999). A novel vascular endothelial growth factor encoded by Orf virus, VEGF-E, mediates angiogenesis via signalling through VEGFR-2 (KDR) but not VEGFR-1 (Flt-1) receptor tyrosine kinases. *EMBO J* **18:** 363-74.

o Michieli P, Mazzone M, Basilico C, Cavassa S, Sottile A, Naldini L *et al* (2004). Targeting the tumor and its microenvironment by a dual-function decoy Met receptor. *Cancer Cell* **6:** 61-73.

o Miller CT, Lin L, Casper AM, Lim J, Thomas DG, Orringer MB *et al* (2006). Genomic amplification of MET with boundaries within fragile site FRA7G and upregulation of MET pathways in esophageal adenocarcinoma. *Oncogene* **25:** 409-18.

o Miller K, Wang M, Gralow J, Dickler M, Cobleigh M, Perez EA *et al* (2007). Paclitaxel plus bevacizumab versus paclitaxel alone for metastatic breast cancer. *N Engl J Med* **357:** 2666-76.

o   Miller KD, Chap LI, Holmes FA, Cobleigh MA, Marcom PK, Fehrenbacher L *et al* (2005). Randomized phase III trial of capecitabine compared with bevacizumab plus capecitabine in patients with previously treated metastatic breast cancer. *J Clin Oncol* **23:** 792-9.

o   Moore MJ, Goldstein D, Hamm J, Figer A, Hecht JR, Gallinger S *et al* (2007). Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. *J Clin Oncol* **25:** 1960-6.

o   Morikawa S, Baluk P, Kaidoh T, Haskell A, Jain RK, McDonald DM (2002). Abnormalities in pericytes on blood vessels and endothelial sprouts in tumors. *Am J Pathol* **160:** 985-1000.

o   Morotti A, Mila S, Accornero P, Tagliabue E, Ponzetto C (2002). K252a inhibits the oncogenic properties of Met, the HGF receptor. *Oncogene* **21:** 4885-93.

o   Morris MC, Deshayes S, Heitz F, Divita G (2008). Cell-penetrating peptides: from molecular mechanisms to therapeutics. *Biol Cell* **100:** 201-17.

o   Motzer RJ, Michaelson MD, Redman BG, Hudes GR, Wilding G, Figlin RA *et al* (2006). Activity of SU11248, a multitargeted inhibitor of vascular endothelial growth factor receptor and platelet-derived growth factor receptor, in patients with metastatic renal cell carcinoma. *J Clin Oncol* **24:** 16-24.

o   Nishino T, Hisha H, Nishino N, Adachi M, Ikehara S (1995). Hepatocyte growth factor as a hematopoietic regulator. *Blood* **85:** 3093-100.

o   Ogawa S, Oku A, Sawano A, Yamaguchi S, Yazaki Y, Shibuya M (1998). A novel type of vascular endothelial growth factor, VEGF-E (NZ-7 VEGF), preferentially utilizes KDR/Flk-1 receptor and carries a potent mitotic activity without heparin-binding domain. *J Biol Chem* **273:** 31273-82.

o   Oikawa T, Onozawa C, Sakaguchi M, Morita I, Murota S (1994). Three isoforms of platelet-derived growth factors all have the capability to induce angiogenesis in vivo. *Biol Pharm Bull* **17:** 1686-8.

o Paez-Ribes M, Allen E, Hudock J, Takeda T, Okuyama H, Vinals F *et al* (2009). Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. *Cancer Cell* **15:** 220-31.

o Pajusola K, Aprelikova O, Pelicci G, Weich H, Claesson-Welsh L, Alitalo K (1994). Signalling properties of FLT4, a proteolytically processed receptor tyrosine kinase related to two VEGF receptors. *Oncogene* **9:** 3545-55.

o Paranjpe S, Bowen WC, Bell AW, Nejak-Bowen K, Luo JH, Michalopoulos GK (2007). Cell cycle effects resulting from inhibition of hepatocyte growth factor and its receptor c-Met in regenerating rat livers by RNA interference. *Hepatology* **45:** 1471-7.

o Park WS, Dong SM, Kim SY, Na EY, Shin MS, Pi JH *et al* (1999). Somatic mutations in the kinase domain of the Met/hepatocyte growth factor receptor gene in childhood hepatocellular carcinomas. *Cancer Res* **59:** 307-10.

o Pasqualini R, Arap W, McDonald DM (2002). Probing the structural and molecular diversity of tumor vasculature. *Trends Mol Med* **8:** 563-71.

o Pennacchietti S, Michieli P, Galluzzo M, Mazzone M, Giordano S, Comoglio PM (2003). Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene. *Cancer Cell* **3:** 347-61.

o Perrotte P, Matsumoto T, Inoue K, Kuniyasu H, Eve BY, Hicklin DJ *et al* (1999). Anti-epidermal growth factor receptor antibody C225 inhibits angiogenesis in human transitional cell carcinoma growing orthotopically in nude mice. *Clin Cancer Res* **5:** 257-65.

o Peschard P, Fournier TM, Lamorte L, Naujokas MA, Band H, Langdon WY *et al* (2001). Mutation of the c-Cbl TKB domain binding site on the Met receptor tyrosine kinase converts it into a transforming protein. *Mol Cell* **8:** 995-1004.

o Ponzetto C, Bardelli A, Maina F, Longati P, Panayotou G, Dhand R *et al* (1993). A novel recognition motif for phosphatidylinositol 3-

kinase binding mediates its association with the hepatocyte growth factor/scatter factor receptor. *Mol Cell Biol* **13:** 4600-8.

o   Ponzetto C, Bardelli A, Zhen Z, Maina F, dalla Zonca P, Giordano S *et al* (1994). A multifunctional docking site mediates signaling and transformation by the hepatocyte growth factor/scatter factor receptor family. *Cell* **77:** 261-71.

o   Prat M, Crepaldi T, Pennacchietti S, Bussolino F, Comoglio PM (1998). Agonistic monoclonal antibodies against the Met receptor dissect the biological responses to HGF. *J Cell Sci* **111 (Pt 2):** 237-47.

o   Prat M, Narsimhan RP, Crepaldi T, Nicotra MR, Natali PG, Comoglio PM (1991). The receptor encoded by the human c-MET oncogene is expressed in hepatocytes, epithelial cells and solid tumors. *Int J Cancer* **49:** 323-8.

o   Pugh CW, Ratcliffe PJ (2003). Regulation of angiogenesis by hypoxia: role of the HIF system. *Nat Med* **9:** 677-84.

o   Rafii S, Meeus S, Dias S, Hattori K, Heissig B, Shmelkov S *et al* (2002). Contribution of marrow-derived progenitors to vascular and cardiac regeneration. *Semin Cell Dev Biol* **13:** 61-7.

o   Reynolds AR, Hart IR, Watson AR, Welti JC, Silva RG, Robinson SD *et al* (2009). Stimulation of tumor growth and angiogenesis by low concentrations of RGD-mimetic integrin inhibitors. *Nat Med* **15:** 392-400.

o   Ribatti D, Nico B, Crivellato E, Roccaro AM, Vacca A (2007). The history of the angiogenic switch concept. *Leukemia* **21:** 44-52.

o   Ribatti D, Vacca A, Presta M (2000). The discovery of angiogenic factors: a historical review. *Gen Pharmacol* **35:** 227-31.

o   Risau W (1997). Mechanisms of angiogenesis. *Nature* **386:** 671-4.

o   Risau W, Drexler H, Mironov V, Smits A, Siegbahn A, Funa K *et al* (1992). Platelet-derived growth factor is angiogenic in vivo. *Growth Factors* **7:** 261-6.

71

o Risau W, Flamme I (1995). Vasculogenesis. *Annu Rev Cell Dev Biol* **11:** 73-91.

o Rocha SF, Adams RH (2009). Molecular differentiation and specialization of vascular beds. *Angiogenesis* **12:** 139-47.

o Safran M, Kaelin WG, Jr. (2003). HIF hydroxylation and the mammalian oxygen-sensing pathway. *J Clin Invest* **111:** 779-83.

o Sandler A, Gray R, Perry MC, Brahmer J, Schiller JH, Dowlati A *et al* (2006). Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. *N Engl J Med* **355:** 2542-50.

o Schmidt C, Bladt F, Goedecke S, Brinkmann V, Zschiesche W, Sharpe M *et al* (1995). Scatter factor/hepatocyte growth factor is essential for liver development. *Nature* **373:** 699-702.

o Schmidt L, Duh FM, Chen F, Kishida T, Glenn G, Choyke P *et al* (1997). Germline and somatic mutations in the tyrosine kinase domain of the MET proto-oncogene in papillary renal carcinomas. *Nat Genet* **16:** 68-73.

o Schroder K, Schutz S, Schloffel I, Batz S, Takac I, Weissmann N *et al* Hepatocyte growth factor induces a pro-angiogenic phenotype and mobilizes endothelial progenitor cells by activating Nox2. *Antioxid Redox Signal*.

o Schwarze SR, Dowdy SF (2000). In vivo protein transduction: intracellular delivery of biologically active proteins, compounds and DNA. *Trends Pharmacol Sci* **21:** 45-8.

o Sengupta S, Gherardi E, Sellers LA, Wood JM, Sasisekharan R, Fan TP (2003). Hepatocyte growth factor/scatter factor can induce angiogenesis independently of vascular endothelial growth factor. *Arterioscler Thromb Vasc Biol* **23:** 69-75.

o Shepherd FA, Rodrigues Pereira J, Ciuleanu T, Tan EH, Hirsh V, Thongprasert S *et al* (2005). Erlotinib in previously treated non-small-cell lung cancer. *N Engl J Med* **353:** 123-32.

o Shojaei F, Wu X, Qu X, Kowanetz M, Yu L, Tan M *et al* (2009). G-CSF-initiated myeloid cell mobilization and angiogenesis mediate

tumor refractoriness to anti-VEGF therapy in mouse models. *Proc Natl Acad Sci U S A* **106:** 6742-7.

o   Silvagno F, Follenzi A, Arese M, Prat M, Giraudo E, Gaudino G *et al* (1995). In vivo activation of met tyrosine kinase by heterodimeric hepatocyte growth factor molecule promotes angiogenesis. *Arterioscler Thromb Vasc Biol* **15:** 1857-65.

o   Stein I, Itin A, Einat P, Skaliter R, Grossman Z, Keshet E (1998). Translation of vascular endothelial growth factor mRNA by internal ribosome entry: implications for translation under hypoxia. *Mol Cell Biol* **18:** 3112-9.

o   Stellrecht CM, Rodriguez CO, Jr., Ayres M, Gandhi V (2003). RNA-directed actions of 8-chloro-adenosine in multiple myeloma cells. *Cancer Res* **63:** 7968-74.

o   Stewart KM, Horton KL, Kelley SO (2008). Cell-penetrating peptides as delivery vehicles for biology and medicine. *Org Biomol Chem* **6:** 2242-55.

o   Suto K, Yamazaki Y, Morita T, Mizuno H (2005). Crystal structures of novel vascular endothelial growth factors (VEGF) from snake venoms: insight into selective VEGF binding to kinase insert domain-containing receptor but not to fms-like tyrosine kinase-1. *J Biol Chem* **280:** 2126-31.

o   Tong CY, Hui AB, Yin XL, Pang JC, Zhu XL, Poon WS *et al* (2004). Detection of oncogene amplifications in medulloblastomas by comparative genomic hybridization and array-based comparative genomic hybridization. *J Neurosurg* **100:** 187-93.

o   Tuck AB, Park M, Sterns EE, Boag A, Elliott BE (1996). Coexpression of hepatocyte growth factor and receptor (Met) in human breast carcinoma. *Am J Pathol* **148:** 225-32.

o   Uehara Y, Minowa O, Mori C, Shiota K, Kuno J, Noda T *et al* (1995). Placental defect and embryonic lethality in mice lacking hepatocyte growth factor/scatter factor. *Nature* **373:** 702-5.

o   van der Voort R, Taher TE, Derksen PW, Spaargaren M, van der Neut R, Pals ST (2000). The hepatocyte growth factor/Met pathway in

development, tumorigenesis, and B-cell differentiation. *Adv Cancer Res* **79:** 39-90.

o van der Voort R, Taher TE, Keehnen RM, Smit L, Groenink M, Pals ST (1997). Paracrine regulation of germinal center B cell adhesion through the c-met-hepatocyte growth factor/scatter factor pathway. *J Exp Med* **185:** 2121-31.

o Verheul HM, Pinedo HM (2007). Possible molecular mechanisms involved in the toxicity of angiogenesis inhibition. *Nat Rev Cancer* **7:** 475-85.

o Vigna E, Pacchiana G, Mazzone M, Chiriaco C, Fontani L, Basilico C *et al* (2008). "Active" cancer immunotherapy by anti-Met antibody gene transfer. *Cancer Res* **68:** 9176-83.

o Waltenberger J, Mayr U, Pentz S, Hombach V (1996). Functional upregulation of the vascular endothelial growth factor receptor KDR by hypoxia. *Circulation* **94:** 1647-54.

o Webb CP, Hose CD, Koochekpour S, Jeffers M, Oskarsson M, Sausville E *et al* (2000). The geldanamycins are potent inhibitors of the hepatocyte growth factor/scatter factor-met-urokinase plasminogen activator-plasmin proteolytic network. *Cancer Res* **60:** 342-9.

o Wilhelm SM, Adnane L, Newell P, Villanueva A, Llovet JM, Lynch M (2008). Preclinical overview of sorafenib, a multikinase inhibitor that targets both Raf and VEGF and PDGF receptor tyrosine kinase signaling. *Mol Cancer Ther* **7:** 3129-40.

o Xie Q, Gao CF, Shinomiya N, Sausville E, Hay R, Gustafson M *et al* (2005). Geldanamycins exquisitely inhibit HGF/SF-mediated tumor cell invasion. *Oncogene* **24:** 3697-707.

o Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J (2000). Vascular-specific growth factors and blood vessel formation. *Nature* **407:** 242-8.

o Yarden Y, Sliwkowski MX (2001). Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* **2:** 127-37.

o You WK, McDonald DM (2008). The hepatocyte growth factor/c-Met signaling pathway as a therapeutic target to inhibit angiogenesis. *BMB Rep* **41:** 833-9.

o Zanetti A, Stoppacciaro A, Marzullo A, Ciabatta M, Fazioli F, Prat M *et al* (1998). Expression of Met protein and urokinase-type plasminogen activator receptor (uPA-R) in papillary carcinoma of the thyroid. *J Pathol* **186:** 287-91.

o Zangari M, Fink LM, Elice F, Zhan F, Adcock DM, Tricot GJ (2009). Thrombotic events in patients with cancer receiving antiangiogenesis agents. *J Clin Oncol* **27:** 4865-73.

o Zaric V, Weltin D, Erbacher P, Remy JS, Behr JP, Stephan D (2004). Effective polyethylenimine-mediated gene transfer into human endothelial cells. *J Gene Med* **6:** 176-84.

o Zarnegar R (1995). Regulation of HGF and HGFR gene expression. *EXS* **74:** 33-49.

o Zelphati O, Szoka FC, Jr. (1996). Intracellular distribution and mechanism of delivery of oligonucleotides mediated by cationic lipids. *Pharm Res* **13:** 1367-72.

o Zhang S, Zhang D, Sun B (2007). Vasculogenic mimicry: current status and future prospects. *Cancer Lett* **254:** 157-64.

o Zhang YW, Su Y, Volpert OV, Vande Woude GF (2003). Hepatocyte growth factor/scatter factor mediates angiogenesis through positive VEGF and negative thrombospondin 1 regulation. *Proc Natl Acad Sci U S A* **100:** 12718-23.

o Zou HY, Li Q, Lee JH, Arango ME, McDonnell SR, Yamazaki S *et al* (2007). An orally available small-molecule inhibitor of c-Met, PF-2341066, exhibits cytoreductive antitumor efficacy through antiproliferative and antiangiogenic mechanisms. *Cancer Res* **67:** 4408-17.

o Zou SM, Erbacher P, Remy JS, Behr JP (2000). Systemic linear polyethylenimine (L-PEI)-mediated gene delivery in the mouse. *J Gene Med* **2:** 128-34.

Acknowledgements