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ORIGINAL ARTICLE

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

AR Cantelmo[1], R Cammarota[1], DM Noonan[1,2], C Focaccetti[1], PM Comoglio[3], M Prat[4,5] and A Albini[1]

[1]*Oncology Research, Science and Technology Pole, Casa di Cura MultiMedica—IRCCS, Milan, Italy;* [2]*Department of Experimental Medicine, University of Insubria, Varese, Italy;* [3]*Institute for Cancer Research and Treatment (IRCC), University of Turin Medical School, Candiolo, Italy;* [4]*Department of Medical Sciences, Università del Piemonte Orientale 'A Avogadro', Novara, Italy and* [5]*BRMA (Biotecnologie per la Ricerca Medica Applicata), Università del Piemonte Orientale 'A Avogadro', Novara, Italy*

Hepatocyte growth factor (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 therapeutic agents. Here we have investigated the antiangiogenic properties of a synthetic peptide mimicking the docking site of the Met carboxyl-terminal tail, which was delivered into the cells by fusion with the internalization sequences from Antennapedia or HIV-Tat. We showed that these peptides inhibit ligand-dependent endothelial 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 signaling. *In vivo*, the peptides inhibited HGF-induced angiogenesis in the matrigel sponge assay and impaired xenograft tumor growth and vascularization in Kaposi's sarcoma. These data show that interference with the Met receptor intracellular sequence impairs HGF-induced angiogenesis, suggesting the use of antidocking site compounds as a therapeutic strategy to counteract angiogenesis in cancer as well as in other diseases.
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Introduction

Angiogenesis contributes to cancer initiation and progression and to several other pathologies, such as chronic inflammation, retinopathies and arthritis (Carmeliet and Jain, 2000). The formation of a tumor-associated vasculature, a process referred to as tumor angiogenesis, is essential for cancer progression and metastasis and has become a clinical target for anticancer therapy. Current clinical approaches all focus on blocking a common hypoxia-associated angiogenic factor, vascular endothelial growth factor (VEGF), by either sequestering the factor itself or inhibiting its receptors. Although effective in slowing tumor progression, tumor escape through a variety of mechanisms, including up-regulation of other angiogenic growth factors, is frequently encountered (Loges *et al*., 2009).

The receptor for the human hepatocyte growth factor (HGF), encoded by *MET* oncogene, has an important physiological role in a complex morphogenetic program known as invasive growth, occurring both during embryonic development and during adult life. In pathological conditions, unregulated HGF-Met signaling can trigger tumor growth and metastasis (Boccaccio and Comoglio, 2006). 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 binding triggers receptor activation through dimerization and auto/trans-phosphorylation of tyrosine residues. Two tyrosine residues within the catalytic loop (Y^{1234}, Y^{1235}) are first to be phosphorylated, leading to full kinase activation (Longati *et al*., 1994). Subsequently, two other tyrosine residues (Y^{1349}, Y^{1356}) 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-γ, cbl, Gab1 and Rho-like GTPases such as RAC1 (Birchmeier *et al*., 2003; Lock *et al*., 2003).

Different strategies to target Met have the aim of inhibiting tumor growth and metastatic dissemination

Correspondence: Dr A Albini, MultiMedica Castellanza, Via Piemonte 70, Castellanza (VA) 21053, Italy or Oncology Research, Science and Technology Pole, Casa di Cura MultiMedica—IRCCS, Via Fantoli 16/15, Milano 20138, Italy.
E-mail: adriana.albini@multimedica.it
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(Comoglio *et al*., 2008). Met 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 (Zou *et al*., 2007; Comoglio *et al*., 2008). 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 (Kim *et al*., 2006; Jun *et al*., 2007), 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 antireceptor monoclonal antibodies are available; these antibodies tend to have agonistic 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). Gene silencing based on antisense, ribozyme and RNA interference techniques have been used experimentally to silence Met and HGF expression (Paranjpe *et al*., 2007; Corso *et al*., 2008; Chu *et al*., 2009).

Finally, peptides interfering with the association of signal transducers to the Met receptor docking site could block the propagation of Met signaling. 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).

On this basis, we investigated whether synthetic Met peptides could interfere with angiogenesis *in vitro* and *in vivo*. The peptide was 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; Gupta *et al*., 2005; Foged and Nielsen, 2008; Morris *et al*., 2008; Stewart *et al*., 2008).

We show that micromolar concentrations of these peptides specifically inhibited HGF-induced *in vitro* migration, invasion and morphogenesis of endothelial cells, interfering with downstream signaling by HGF. Comparison with the inhibitor PHA-665752 showed that the peptides exerted greater inhibitory effects, yet no toxicity, whereas PHA-665752 showed substantial toxicity. *In vivo*, the peptides impaired HGF-dependent angiogenesis and significantly inhibited the growth of Kaposi's sarcoma xenograft, as well as vascularization. These data suggest a possible pharmacological mechanism to interfere with Met-mediated angiogenesis.

Results

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 1). An 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.

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 (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays was observed at doses of 10 μM Antp-Met already after 24 h of treatment (Figure 2a). 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 (Figures 2b and c). At higher concentrations (20 and 50 μM) they displayed a cytostatic activity starting from 24 h 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-h time point when we exposed endothelial cells to micromolar concentrations (5 and 10 μM) of PHA-665752 (Figure 2d). Lower PHA-665752 concentrations did not exert any significant reduction in 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,

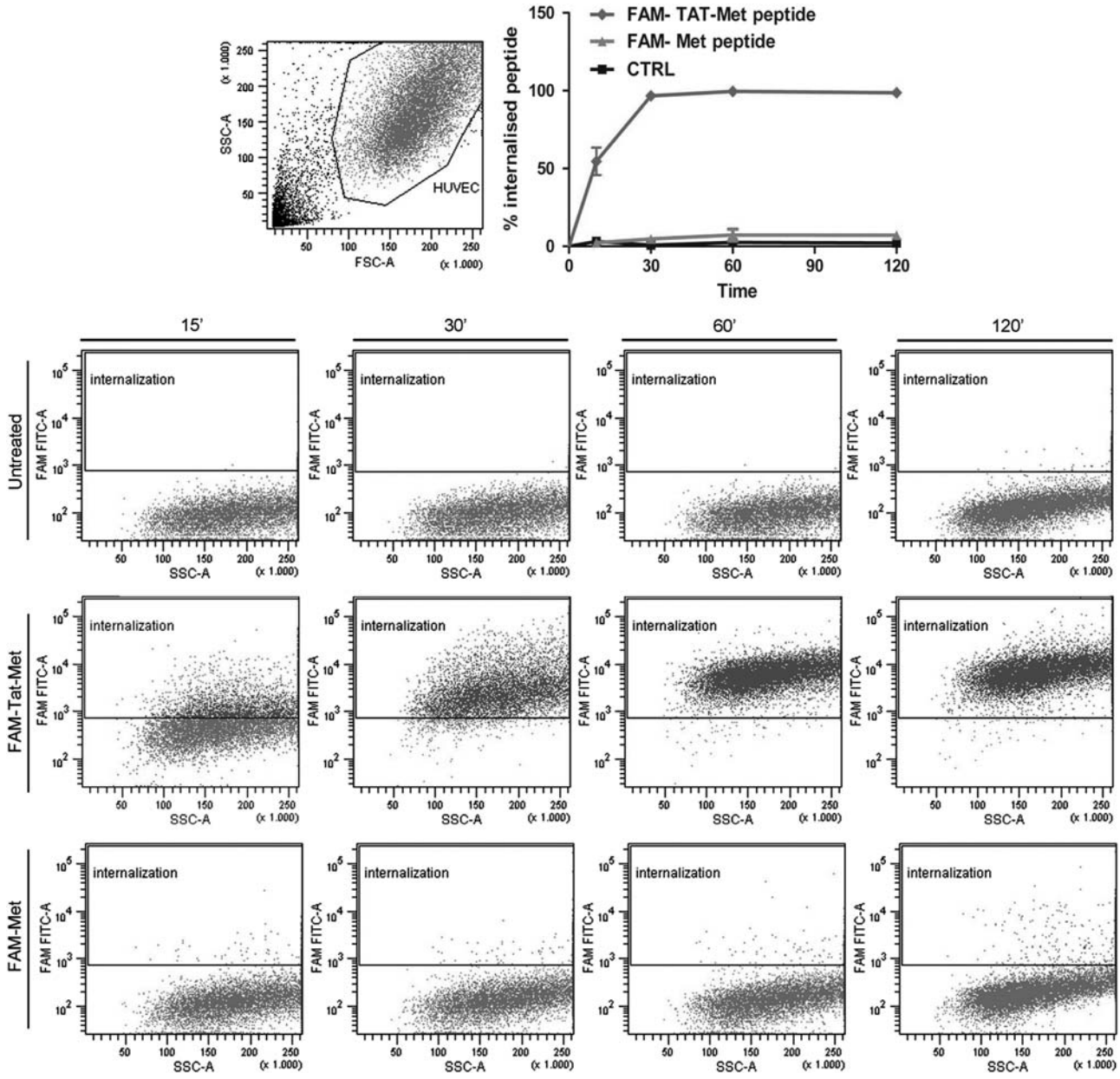


Figure 1 The Tat-Met peptide enters into HUVECs. Tat-Met was conjugated at the N-terminus with FAM. HUVECs (1×10^5) were incubated for different periods of time with 10 μM peptide, washed twice and analyzed by fluorescence-activated cell sorting. Labeled Met peptide devoid of the cell-penetrating peptide motif and untreated cells (CTRL) were used as controls. In the lower panels, representative dot plots of peptide internalization percentage are shown.

whereas PHA-665752 required relatively high concentrations to attain comparable effects.

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

The possibility that the Met inhibitors used could exert toxicity or induce endothelial cell apoptosis was evaluated by cytofluorimetric analysis. After only 2 h of treatment of endothelial cells in the presence of HGF with 250 nM PHA-665752, approximately 60% of the cells (Figure 3a) were already undergoing apoptosis (both Annexin V and 7-amino-actinomycin D positive). In contrast, cells treated with the peptides, even at highest concentrations, showed a high percentage (about 95%) of viable endothelial cells and no apoptosis (Figure 3a). The toxicity of PHA-665752 was observed even in the presence of serum (fetal bovine serum (FBS)) with clear dose-dependent effects (Figure 3b). These data suggest that the growth inhibition exerted by PHA-665752 may be due to cytotoxicity and apoptosis.

The 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

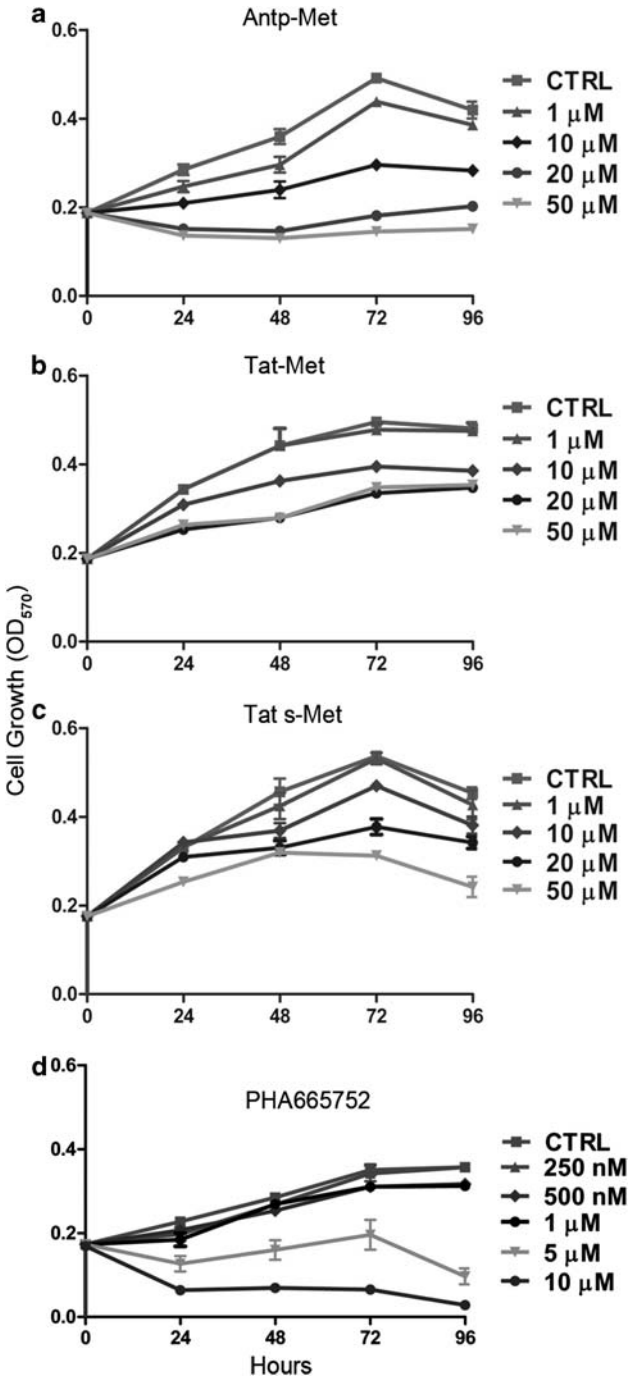


Figure 2 The Met docking site peptide inhibits HUVEC proliferation (MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)assay). HUVECs were plated at low density, and then starved in low serum. Cell proliferation was evaluated at fixed times after the addition of HGF (50 ng/ml) +/− Antp-Met (**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 a marked reduction in cell numbers starting from 24 h at micromolar concentrations (5 and 10 μM), whereas lower concentrations did not exert a significant reduction of proliferation with respect to the control. Means ± s.e.m. are shown ($P<0.05$).

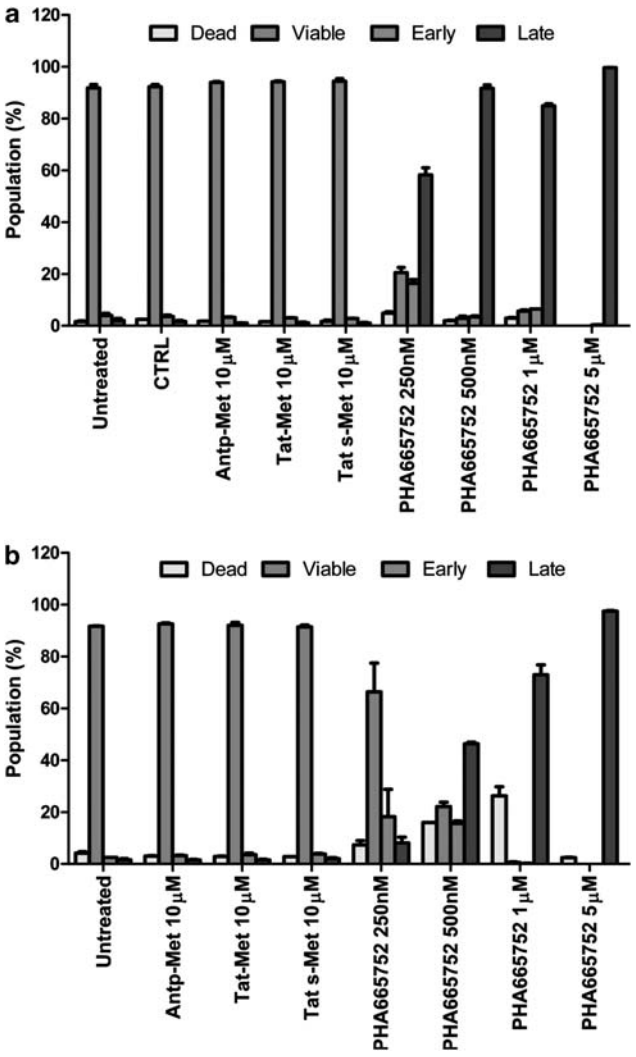


Figure 3 Met docking site peptides do not induce toxicity or apoptosis in endothelial cells. In the apoptosis assay, HUVECs were plated onto six-well plates and were treated with either the Antp-Met peptide (10 μM), Tat-Met peptide (10 μM) or Tat s-Met peptide (10 μM) or with increasing concentrations of PHA-665752 (250 and 500 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.

HGF-dependent HUVEC motility and invasion. Under the effect of HGF (50 ng/ml), the number of migrated cells in a Boyden chamber assay doubled; a non-cytotoxic dose of 10 μM peptides abrogated this migration (Figure 4a; $*P<0.05$). No significant differences in migration-inhibitory activity were found among the different peptides. We observed equivalent inhibitory effects with PHA-6657525 only in the presence of the highest (cytotoxic) concentrations (5 and 10 μM)

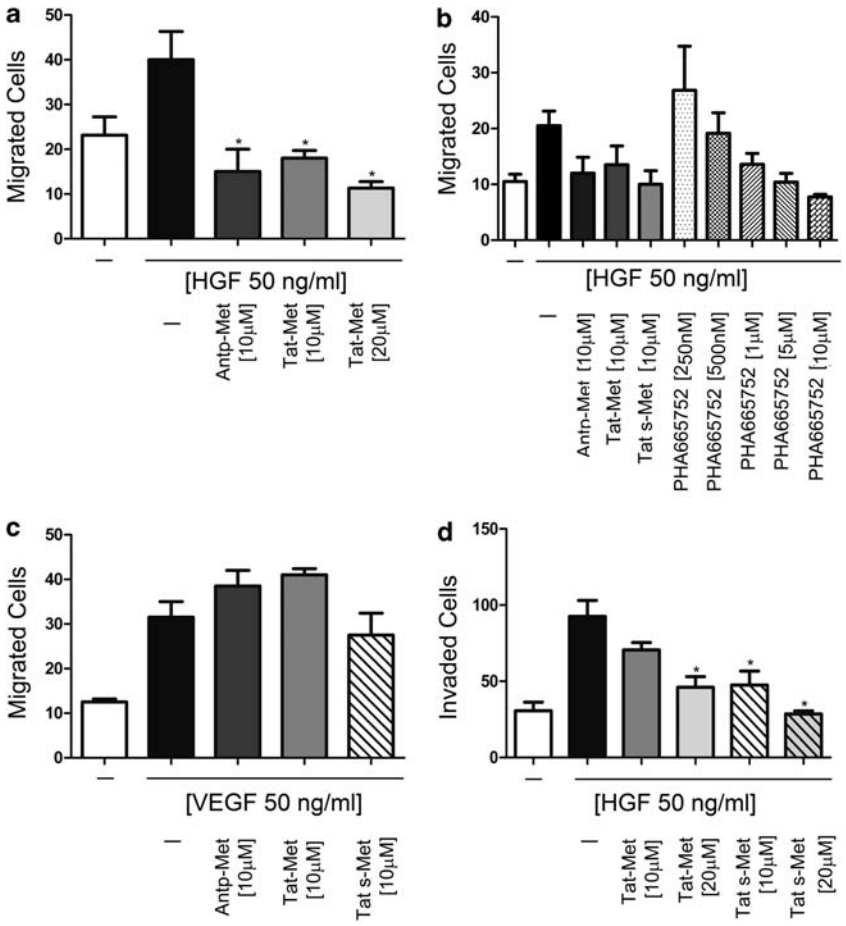


Figure 4 The Met docking site peptide strongly inhibits HGF-dependent HUVEC migration and invasion. In the migration assay, HUVECs were seeded into the upper compartment of Boyden chambers in the presence or absence of Antp-, Tat- and Tat s-Met peptides or PHA-665752, whereas chemoattractants (50 ng/ml 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 (*$P<0.05$).

(Figure 4b). The Met peptides did not inhibit VEGF-dependent cell migration (Figure 4c), thus showing clear specificity.

In an invasion assay performed in Boyden chambers with filters pre-coated with a reconstituted basement membrane (matrigel), treatment with Tat-Met or Tat s-Met significantly reduced the HGF-induced invasion (Figure 4d). 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 4d).

The Met docking site peptide inhibits endothelial morphogenesis in vitro

Human umbilical vein endothelial cells can organize into capillary-like structures when cultured on a thick matrigel layer in the presence of angiogenic factors. The Met peptides interfered with HGF-induced HUVEC morphogenesis with the same efficacy shown in inhibiting cell proliferation (Supplementary data). Antp-Met was effective at 10 μM (Supplementary data), whereas Tat-Met and Tat s-Met were effective at 20 μM. Antp-Met did not affect FBS-dependent network formation, showing specificity of the inhibitory effect (Supplementary data). PHA-665752 interfered with HGF-dependent tube formation to a similar extent as that of the peptides only at cytotoxic micromolar concentrations (Supplementary data).

The Met docking site peptide inhibits HGF-dependent downstream signaling

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 signaling pathways, the ERK1/2 and the phosphoinositide 3-kinase

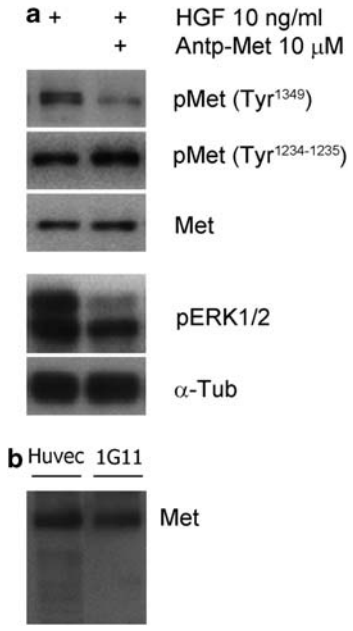


Figure 5 The Met docking site peptide inhibits the HGF-dependent downstream signaling. Quiescent HUVECs were pre-treated or not for 2 h with Antp-Met before stimulation with 10 ng/ml HGF for 15 min, and then lysed and processed for Western blot analysis. Blots were probed with anti-phospho Met (tyrosine 1349), anti-phospho Met (tyrosines 1234–1235), anti-phospho ERK1/2, anti-Met and anti-α-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.

(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. Although phosphorylation on tyrosines 1234−1235, localized in catalytic loop, was not affected, phosphorylation on tyrosine 1349, localized in the carboxyl-terminal docking site, showed a 70% reduction when Antp-Met was added to cells 2 h before stimulation with 10 ng/ml HGF (Figure 5a). Phosphorylation of ERK1/2, which is mainly involved in transducing proliferation and motility responses (Prat *et al*., 1998; Birchmeier *et al*., 2003), was also impaired by the Antp-Met peptide (Figure 5a). 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 signaling, but do not affect phosphorylation of the tyrosines located in the activation loop.

The 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 (50 U/ml) promoted a hemorrhagic vascularization of the matrigel sponge, which was clearly detectable at 4 days post-implantation (Figure 6a). 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 6b). 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 6b). Hematoxylin- and eosin-stained sections of pellets showed that the extensive vascularized areas in mice treated with HGF and heparin were substantially reduced in mice treated with the Met peptides (10 μM) (Figures 6Ca, 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 (Figures 6Ca′, b′, c′).

The Met docking site peptide inhibits the growth and vascularization of Kaposi's sarcomas in nude mice
We next examined whether the peptides were able to 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 subcutaneously every second day until termination of the experiment. Treatment with the Tat s-Met peptide clearly inhibited tumor growth (Figure 7a). Hematoxylin–eosin staining of sections of tumors showed extensive areas of vascularization in control tumors, whereas extensive necrotic areas and strongly reduced vascularization were found in tumors of animals receiving peptides (Figures 7Ba, b). Immunohistochemical staining indicated that in peptide-treated tumors CD11b/c-positive inflammatory cells were significantly reduced (Figures 7Ba′, b′). To confirm the reduction of vascularization in tumors treated with peptide, we performed an immunofluorescence analysis for the endothelial marker von Willebrand (Figures 7Cb, b′) and we observed a higher number of von Willebrand-positive cells (Figure 7Cb) in control tumors than in tumors treated with Tat s-Met (Figure 7Cb′). We observed colocalization of the Met receptor and the von Willibrand endothelial marker in the tumors (Figures 7Cc, c′), suggesting that murine tumor endothelial cells express Met. This was consistent with the presence of Met (Figure 5b) 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), was injected in the peri-tumor area every second day after the tumor was established. Again, the peptide significantly inhibited tumor growth, whereas the scrambled peptide had no effect on tumor growth (Figure 7D). The central necrotic areas (Figures 7Ea, b) and the strong inflammatory infiltrate (Figures 7Ea′, b′) again were strongly reduced in the presence of the Met peptide as compared with 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.

Discussion

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 a promising target (Folkman, 1985; Carmeliet and Jain, 2000; Ferrara, 2004; 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 downregulating 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 antiangiogenic therapy (You and McDonald, 2008).

Structural and functional integrity of endothelial cells is a fundamental factor for vessel homeostasis and function. Alterations in the 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 to induce vascular dysfunction. Several small-molecule drugs targeting Met and other kinases show toxicity; biological molecules could be used to obtain higher levels of specificity and lower toxicity in Met inhibition.

Here we describe the antiangiogenic properties of synthetic cell-permeable peptides containing the carboxyl-terminal tail of Met in the absence of cytotoxicity or apoptosis induction. These peptides inhibit HGF-induced proliferation, migration, invasiveness and capillary-like 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 that were 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

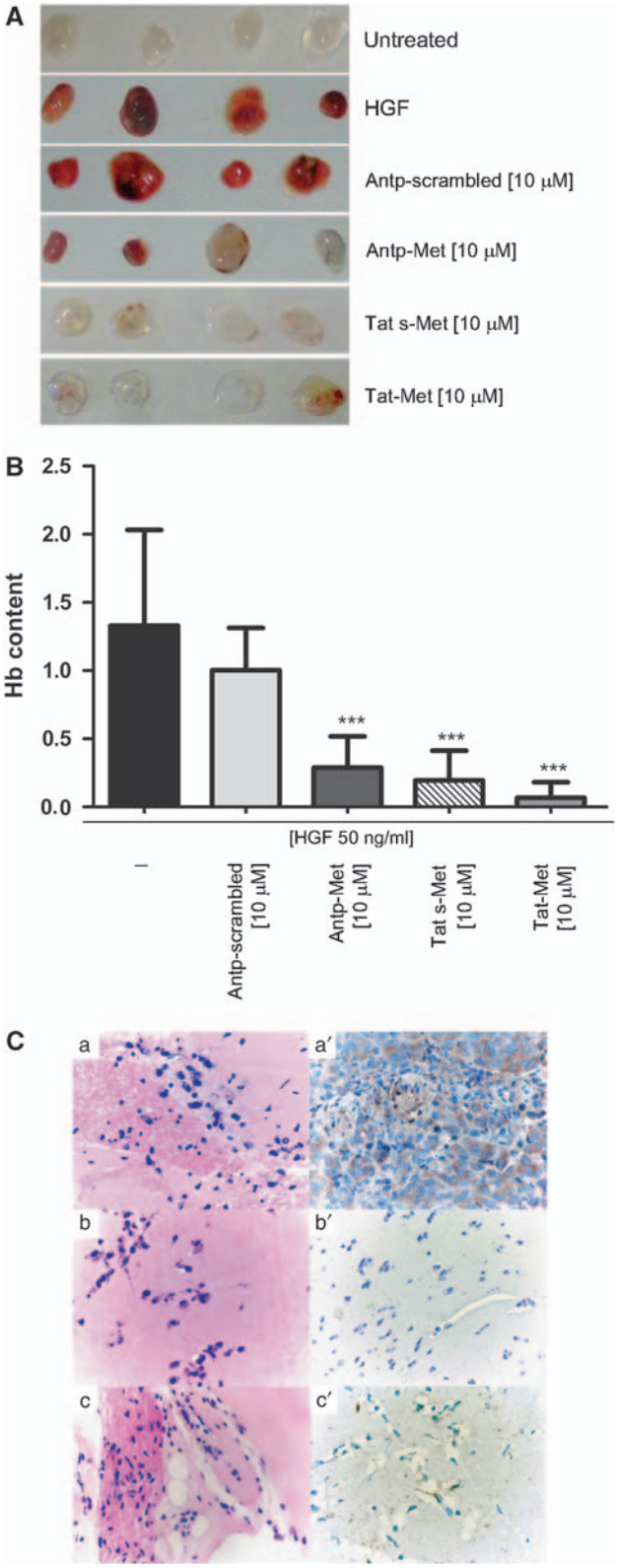


Figure 6 The Met docking site peptide inhibits *in vivo* angiogenesis. A cocktail of HGF (50 ng/ml) and heparin (50 U/ml) promoted a hemorrhagic vascularization of the matrigel sponge clearly detectable at 4 days post-implantation. HGF-induced vascularization of the subcutaneously injected matrigel implants 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′)) (×400 magnification).

(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 have 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 the role of Met in endothelial cells (Figure 6b), the co-expression with the endothelial marker *in vivo* (Figure 7b) 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 (Ponzetto *et al*., 1994; Birchmeier *et al*., 2003), consistent with previous *in vitro* studies in epithelial cells (Bardelli *et al*., 1999). The Met-specific peptides were delivered into cells by fusion with the internalization sequences from the Antp homeodomain 3 or the HIV-Tat basic domain, which proved to act efficiently as transporters across membranes (Deshayes *et al*., 2005; Gupta *et al*., 2005; Foged and Nielsen, 2008; Morris *et al*., 2008; Stewart *et al*., 2008). A similar peptide fused to the Tat internalizing sequence enhanced *in vitro* chemosensitivity of glioma cells to a *cis*-platinum derivative (Lou *et al*., 2009). The three peptides containing the Met docking site coupled to different internalizing sequences showed 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 Y^{1349}, which binds the p85 regulatory subunit of phosphoinositide 3-kinase (Graziani *et al*., 1991; Ponzetto *et al*., 1994; Bardelli *et al*., 1999; Lou *et al*., 2009), mainly involved in transducing HGF-dependent motogenic and antiapoptotic responses (Ponzetto *et al*., 1993; Khwaja *et al*., 1998; Giordano *et al*., 2000; Deregibus *et al*., 2003). Phosphorylation of the MAPK ERK1/2, whose activation is required for the proliferative and invasive responses (Ponzetto *et al*., 1994; Prat *et al*., 1998; Birchmeier *et al*., 2003; Bardelli *et al*., 2005), was impaired. Although strongly interfering with the activation of the tyrosines located in the docking site, the peptide had no effect on phosphorylation of Y^{1234}, Y^{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 signaling by either anti-VEGF antibodies or multitargeted receptor tyrosine kinase inhibitors (Folkman, 1985; Carmeliet and Jain, 2000; Ferrara, 2004; 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 (Pennacchietti *et al*., 2003; Ebos *et al*., 2009; Paez-Ribes *et al*., 2009). The fact that Met is both a pro-angiogenic factor and an oncogene suggested that it may be an optimal target for anticancer treatment, simultaneously inhibiting angiogenesis and oncogenesis (Michieli *et al*., 2004) and potentially complementing VEGF targeted antiangiogenic 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.

Materials and methods

Reagents

Murine recombinant VEGF-A, human recombinant VEGF-A165, murine recombinant tumor necrosis factor-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 at their N-terminus were used to evaluate internalization. Peptide sequences are reported in Table 1.

Cell cultures

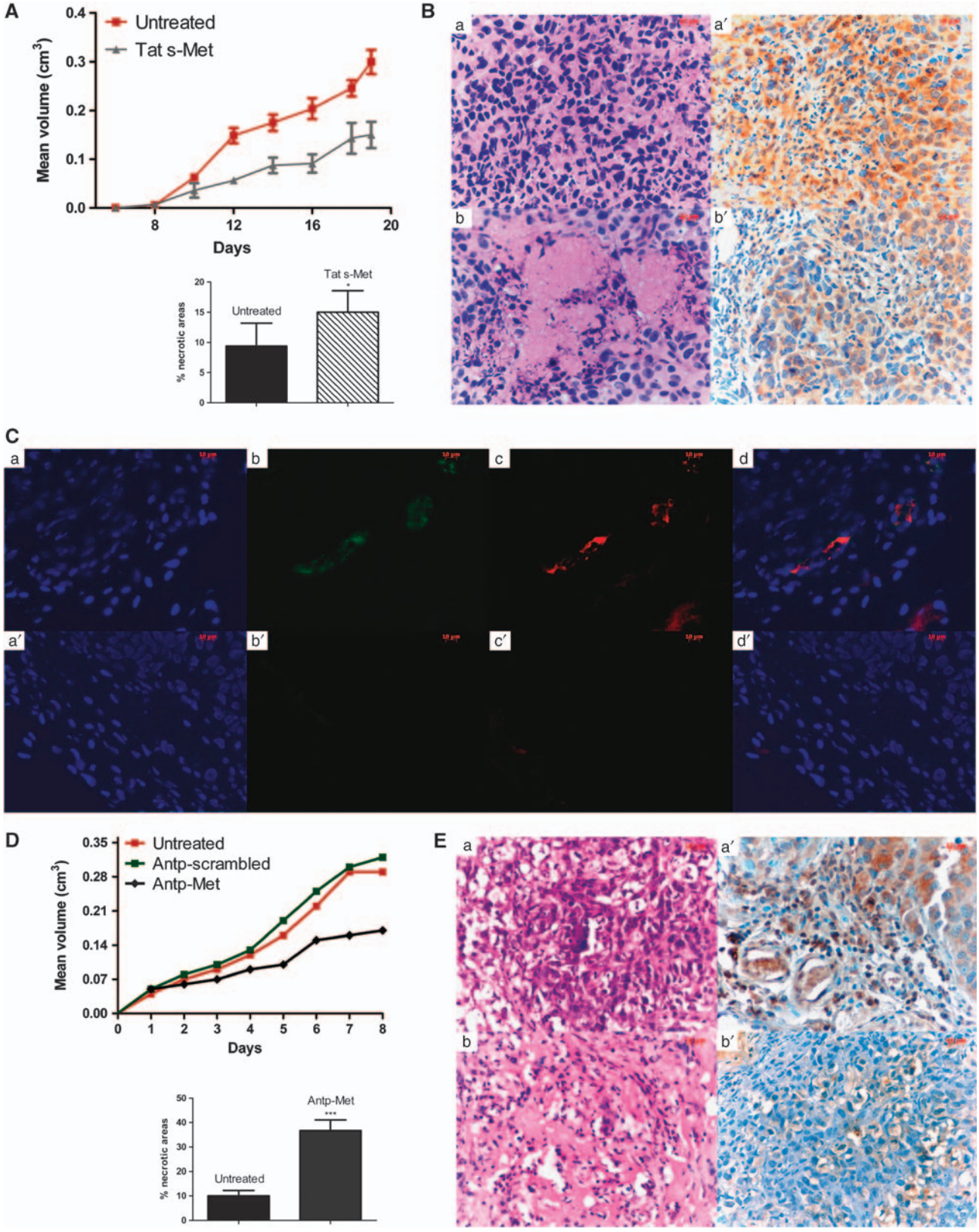
Human umbilical vein endothelial cells were purchased from Promo Cell (Heidelberg, Germany) and grown on 1% gelatin-coated tissue culture plates in M199 endothelial growth medium (Sigma, St Louis, MO, USA), supplemented with 10% heat-inactivated FBS, 1% L-glutamine, fibroblast growth factors (1 μg acid-fibroblast growth factor plus 1 μg basic-fibroblast growth factor /100 ml), epidermal growth factor (1 μg/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).

Cytofluorimetric analysis

Human umbilical vein endothelial cells (6×10^5) 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), 1×10^5 HUVECs were plated onto 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 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 (0.01 M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (pH 7.4); 0.14 M NaCl; 2.5 mM $CaCl_2$). 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 µl 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.

In vitro *cell viability*
Human umbilical vein endothelial cell viability was evaluated in the MTT assay. Cells (1000/well) were seeded into 96-multiwell plates in complete medium and, 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 different periods of incubation (24, 48, 72 and 96 h), plates were processed and absorbance read at 570 nm.

Migration, invasion and morphogenesis assays
These assays were performed in modified Boyden chambers, as described previously (Albini *et al*., 1987; Albini and Benelli, 2007). HUVECs (5×10^4) were washed with PBS, resuspended in serum-free medium and placed in the upper compartment. Chemoattractants (50 ng/ml HGF or 10 ng/ml VEGF) in serum-free M199 medium were added in the lower compartment and the different peptides or PHA-665752 at different concentrations added. Pore-size polycarbonate filters (12 µM) were pre-coated with collagen (50 µg/ml) or matrigel (1 mg/ml). After 6 h (migration) or overnight (invasion) incubation, the filters were recovered, cells on the upper surface mechanically removed and cells migrated to or invaded the lower filter surface fixed with absolute ethanol and stained with toluidine blue. Cells were counted in a double-blind manner in eight consecutive fields each with a light microscope. All experiments were performed three times in triplicate. The *in vitro* morphogenesis assay was performed as described by Grant *et al*. (1989) in the presence of 50 ng/ml HGF or 10% FBS, and with the peptides or PHA-665752 in increasing concentrations. After 6 h incubation on matrigel, the dimensional organization of the cells was examined under an inverted microscope (Zeiss, Oberkochen, Germany), equipped with CCD optics and a digital analysis system.

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.

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 hemoglobin

Table 1 Peptide sequences

Antp-Met	RQIKIWFQNRRMKWKK IGEHYVHVNATYVNV KCVA
Tat-Met	GRKKRRQRRRPP IGEHYVHVNATYVNVKCVA
Tat s-Met	RKKRRQRRR IGEHYVHVNATYVNVKCVA
Antp-scrambled	RQIKIWFQNRRMKWKK IVAVNCGV-HYEHTNVKVYA
Met	IGEHYVHVNATYVNVKCVA

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.

Figure 7 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 µl 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. The inset shows a quantitative analysis of the areas necrosis (as a percentage) in control and Tat s-Met-treated tumors (mean ± s.e.; $*P<0.001$). (**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 strongly 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 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. The inset shows a quantitative analysis of the areas necrosis (as a percentage) in control and Antp-Met-treated tumors (mean ± s.e.; $***P<0.001$). (**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 tumor-associated inflammation (b′) as compared with controls (a′) (× 400 magnification).

concentration was calculated from a standard calibration curve, after spectrophotometric analysis at 540 nm.

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*., 1997, 2001). Tat s-Met peptide was injected with cells (300 μl final volume of 10 μM peptide corresponding to 34 μg/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 μg/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 animal was 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).

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 3,3′-diaminobenzidine (Dako REAL EnVision system, Peroxidase/diaminobenzidine+, 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).

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.

Conflict of interest

The authors declare no conflict of interest.

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