Vasculotide reduces endothelial permeability and tumor cell extravasation in the absence of binding to or agonistic activation of Tie2

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Abstract

Angiopoietin-1 (Ang1) activation of Tie2 receptors on endothelial cells (ECs) reduces adhesion by tumor cells (TCs) and limits junctional permeability to TC diapedesis. We hypothesized that systemic therapy with Vasculotide (VT)—a purported Ang1 mimetic, Tie2 agonist—can reduce the extravasation of potentially metastatic circulating TCs by similarly stabilizing the host vasculature. In vitro, VT and Ang1 treatments impeded endothelial hyperpermeability and the transendothelial migration of MDA-MB-231-LM2-4 (breast), HT29 (colon), or SN12 (renal) cancer cells to varying degrees. In mice, VT treatment inhibited the transit of TCs through the pulmonary endothelium, but not the hepatic or lymphatic endothelium. In the in vivo LM2-4 model, VT monotherapy had no effect on primary tumors, but significantly delayed distant metastatic dissemination to the lungs. In the post-surgical adjuvant treatment setting, VT therapeutically complemented sunitinib therapy, an antiangiogenic tyrosine kinase inhibitor which limited the local growth of residual disease. Unexpectedly, detailed investigations into the putative mechanism of action of VT revealed no evidence of Tie2 agonism or Tie2 binding; alternative mechanisms have yet to be determined.

Keywords angioptoin; metastasis; Tie2; tumor cell extravasation; vascular permeability

Subject Categories Cancer; Vascular Biology & Angiogenesis

Introduction

Metastatic disease—as opposed to primary tumors—accounts for 90% of cancer-related mortality (Steeg, 2012). Most cancer drugs are selected from preclinical studies based on their potency at inhibiting primary tumor growth, and brought into clinical trials with the rationale that they will similarly inhibit the growth of metastases (Francia et al, 2011; Guerin et al, 2013). The inadequacy of this approach has been reviewed and editorials have highlighted the need for new anti-metastatic therapies that block not just the growth (progression) but also the spread or formation (incidence) of metastases (Steeg, 2012).

The metastatic process comprises a cascade of events (Talmadge & Fidler, 2010; Hanahan & Weinberg, 2011): stromal invasion from a localized tumor; intravasation of tumor cells (TCs); their systemic circulation and arrest in distant capillary beds; TC extravasation into the host organ parenchyma; and overt colonization as micrometastases grow into macrometastases. In clinically relevant settings, the temporal window of TC extravasation is asynchronous and wide: Primary tumors shed millions of TCs per gram of tumor into the blood circulation every day (Bockhorn et al, 2007), and metastases themselves can metastasize to tertiary sites or ‘self-seed’ back to primary sites (Comen et al, 2011). Surgical trauma associated with primary tumor resections can also sometimes paradoxically fuel metastatic spread—for example, by mechanically dislodging tumor cells into the circulation and by inducing the production/release of inflammatory and angiogenic cytokines that promote metastatic seeding and progression (Goldfarb & Ben-Eliyahu, 2006).

Tumor cell extravasation is regulated by many cytokines, including angiopoietin-1 (Ang1), a guardian of EC quiescence (Augustin et al, 2009; Huang et al, 2010), as well as angiopoietin-2 (Ang2) and vascular endothelial growth factor (VEGF), the cooperative initiator and driver of angiogenesis (Huang et al, 2010; Felcht et al, 2012). It was found that VEGF stimulates, Ang2 potentiates, while Ang1 counteracts the TNF-α/NF-κB-mediated EC surface expression of ICAM-1, VCAM-1, and E-selectin—which facilitate the adhesion and migration of TCs across the endothelium (Kim et al, 2001b; Fiedler et al, 2006; Miles et al, 2008; Huang et al, 2010). VEGF also stimulates, while Ang1 counteracts Src-mediated destabilization of paracellular VE-cadherin junctions and IP3/eNOS-mediated calcium influxes in ECs—which together cause vascular hyperpermeability and lowered resistance to TC diapedesis (Gamble et al, 2000; Gavard et al, 2008; Le Guelte et al, 2011; Koh, 2013).
Angiopoietins have become targets of growing interest in the development of cancer therapeutics (Huang et al., 2010). Based on the above, both anti-Ang2 and pro-Ang1 strategies should have the potential to impede TC extravasation by reducing TC-EC adhesion and vascular permeability.

Preclinically, anti-Ang2 agents have largely been studied in the context of primary tumor growth, often demonstrating additive anti-angiogenic effects when combined with VEGF pathway inhibitors (Brown et al., 2010; Doppalapudi et al., 2010; Koh et al., 2010; Huang et al., 2011; Mazzieri et al., 2011; Schaefer et al., 2011; Holopainen et al., 2012; Leow et al., 2012). Some have further shown anti-metastatic activity preclinically through vascular-stabilizing systemic effects (Holopainen et al., 2012).

In the context of suppressing primary tumor growth by targeting the tumor vasculature, concurrent Ang1 inhibition was thought to augment the activity of Ang2-specific inhibition (Falcon et al., 2009; Coxon et al., 2010), but metastatic disease was not modeled in these preclinical studies. In the clinic, the dual Ang2/Ang1-neutralizing peptibody, trebananib (AMG386), recently failed to meet its secondary endpoint of overall survival, despite improving progression-free survival (PFS) in earlier primary endpoint analysis (Monk et al., 2014), when combined with paclitaxel for patients with recurrent platinum-sensitive ovarian cancer in the phase III TRINOVA-1 trial. Trebananib had also failed in phase II trials involving metastatic gastro-esophageal (Eatock et al., 2013), colorectal (Peeters et al., 2013), and clear-cell renal (Rini et al., 2012) carcinomas. This lack of clinical efficacy raises a question: Could concurrent Ang1 inhibition actually be compromising the anti-metastatic efficacy of Ang2-specific inhibition by destabilizing systemic blood vessels to promote distant metastatic spread, despite the expected additive benefit in local tumor growth suppression (Cascone & Heymach, 2012)? This is plausible, given the evidence that Ang1 inhibition can lead to systemic dysfunction of vessels in healthy tissues (Thomas et al., 2013).

On the other hand, preclinical evidence is conflicting on whether Ang1 overexpression by genetic approaches (Ahmad et al., 2001; Hawighorst et al., 2002; Tian et al., 2002; Machein et al., 2004; Holopainen et al., 2009; Huang et al., 2009; Schulz et al., 2011) has positive or negative effects on tumor growth and metastasis. Recently, the subcutaneous administration of a recombinant Ang1 variant protein (‘Ang-F1-Fc-F1’, also called ‘BowAng1’) on its own had no effect on primary tumors, but as a concurrent treatment diminished the anti-angiogenic and anti-tumor efficacy of an anti-Ang2 antibody (Daly et al., 2013). This suggests that Ang1 supplementation may be counterproductive, even if not detrimental, in terms of controlling localized primary tumor growth; however, this aforementioned study did not model metastatic disease, where the impact may be fundamentally different (Guerin et al., 2013).

We hypothesize that systemic pro-Ang1 therapy can inhibit TC extravasation and metastatic spread, by reducing TC-EC adhesion and vascular permeability. In this study, we sought to test these hypotheses using Vasculotide (described below) as our candidate Ang1-mimetic vascular-stabilizing agent, with or without an anti-angiogenic VEGF pathway inhibitor, in in vitro models of tumor cell extravasation and in vivo models of metastasis.

The two angiopoietin ligands share the same cognate tyrosine kinase receptor, Tie2— with Ang1 being the main agonist, while Ang2 often acts as a competitive antagonist and sometimes as a partial agonist (Thurston et al., 2005; Bogdanovic et al., 2006; Augustin et al., 2009; Yuan et al., 2009; Thurston & Daly, 2012). Their monomeric structures are highly similar, but Ang1 predominantly exists in higher-order multimeric forms, while Ang2 mainly exists in dimeric form (Davis et al., 2003; Cho et al., 2004; Kim et al., 2005). Minimally tetrameric oligomerization of Ang1 was thought to be a requirement of its activity as a Tie2 agonist, while monomeric and dimeric Ang1 antagonized Tie2 activity in Ang2-like fashion (Davis et al., 2003).

For therapeutic use, recombinant variants or mimetics of multi- meric Ang1 have been engineered, including Ang1* and Ang-F1-Fc- F1/Bow-Ang1 (Davis et al., 2003; Daly et al., 2013), MAT-Ang1 and COMP-Ang1 (Cho et al., 2004; Koh, 2013). Unlike these Ang1 variants, Vasculotide (VT) does not adopt the globular, 215-amino-acid-long, Tie2-binding ‘fibrinogen-like domains’ of native Ang1 (Davis et al., 2003; Cho et al., 2004). The development of VT was inspired by a 2004 paper, where several heptapeptides—with no sequence homology to Ang1—were identified to have Tie2-binding potential through an ELISA screen of a phage-displayed peptide library (Tournaire et al., 2004). Among them was ‘T7’ (HHHRHHSF), which in its synthetic free form did not inhibit Ang1 or Ang2 binding to Tie2 in competition assays (Tournaire et al., 2004). By conjugating together four copies of ‘T7’, using an avidin backbone in the first-generation design, VT was developed with the aim of tetramerically binding and clustering Tie2 receptors in an ‘Ang1-like’ manner to activate Tie2 signaling (Van Slyke et al., 2009). The current generation of VT is PEGylated (Supplementary Fig S1): It employs a 4-arm maleimide-functionalized polyethylene glycol (PEG) backbone to link together four cysteine-capped T7 peptides, that is, CHHHRHSF (‘T7c’). This PEGylated VT reportedly activates Tie2 phosphorylation and induces Ang1-like cellular and physiological responses. For instance, in endotoxemic mice, VT prevented inflammatory induction of lung vascular hyperpermeability by preserving VE-cadherin-mediated EC junctions (David et al., 2011). In an in vivo model of abdominal sepsis, VT reduced intraperitoneal leukocyte influx through suppression of pro-inflammatory cytokines (e.g., TNF-α, IL-6) and endothelial adhesion molecules (e.g., ICAM-1 and VCAM-1) (Kumpers et al., 2011). In a mouse model of skin toxicity from cancer radiotherapy, VT promoted wound healing by reducing irradiation-induced inflammation (e.g., neutrophil recruitment) and microvascular damage (Korpela et al., 2014). The objective of our study was to harness these potentially promising vascular-stabilizing and anti-inflammatory properties of VT in developing an Ang1-mimetic therapeutic strategy for inhibiting early stages of metastatic spread.

**Results**

Vasculotide has permeability-limiting effects on endothelial cells and inhibits tumor cell extravasation in vitro

Vasculotide was previously shown to preserve endothelial barrier integrity when such cells are stimulated with sepsis-related mediators of vascular hyperpermeability, including thrombin (David et al., 2011). Thrombin is also a metastasis-associated factor that can promote endothelial adhesion and diapedesis of TCs (Nierodzik & Karpatkin, 2006).
Using in vitro modified Boyden chamber assays, where insert filter membranes were lined by confluent human microvascular ECs of either lung or dermal blood vessel origin (HMVEC-LBl, Fig 1A–C; HMVEC-DBl, Fig 1D and E), we observed that VT treatment was able to counteract thombin-stimulated increases in transendothelial permeability of FITC-dextran ($P < 0.05$, Fig 1A and D) and showed trends of reducing thombin-stimulated migration of CMTPX-labeled TCs (Fig 1B, C and E).

Several structural components of VT were additionally tested as controls (Supplementary Fig S1): ‘T7c’ refers to non-PEGylated CHHHRHSF peptides, and ‘PEG-Cys’ refers to the PEG backbone alone. While these structural components individually contributed partial effects, the intact structure of VT appeared to be necessary for full anti-permeability effect (Fig 1D). Moreover, saturation of VT’s binding targets by 100-fold molar excess of T7c peptides dampened the effects of VT (Fig 1D and E), suggesting that VT function is at least partially dependent on its T7 moieties.

**Breast cancer metastasis model: Vasculotide inhibits experimental LM2-4luc metastasis to the lungs but not lymphatics**

We next assessed the anti-metastatic potential of VT in vivo using three models of ‘experimental metastasis’. By injecting a fixed number of human breast (LM2-4luc), colon (HT29luc), or renal (SN12luc) cancer cells directly into the venous circulation of SCID mice, we modeled specifically the later steps of the ‘metastasis cascade’—TC extravasation and metastatic colonization—within specific host organs particularly susceptible to each cancer type.

We showed previously that sunitinib (SU) treatment prior to intravenous (IV) inoculation of LM2-4luc cells, through pre-conditioning of the host environment, can lead to a promotion of experimental metastasis, especially in the lungs (Ebos et al, 2009)—results confirmed by others (Chung et al, 2012; Welta et al, 2012). Here, we tested whether concurrent VT therapy can be used to reverse this pro-metastatic potential of SU in the same model. LM2-4luc is a metastatically aggressive luciferase-tagged derivative of the MDA-MB-231 cell line that was derived through serial in vivo selection of lung metastases (Munoz et al, 2006; Francia et al, 2011). Early while passages of LM2-4luc maintained a high propensity for lung colonization, later passages of LM2-4luc reverted to its parental bias for lymphatic colonization (F.T. Wu, C.R. Lee and R.S. Kerbel, unpublished observations).

Figure 2 panels A–D summarize an experiment where a predominance of lung-specific metastases developed after IV inoculation of early-passage LM2-4luc cells into SCID mice, so that the main reason for sacrifice or mortality was labored breathing. In this case, long-term 250 ng/2d VT monotherapy conferred a significant survival benefit ($P = 0.01$), extending median survival by ~20% (Fig 2A). In vivo bioluminescent imaging (IVBI) recorded a trend of reduced lung metastases with VT monotherapy (Fig 2B–D). As a concurrent therapy to SU (60 mg/kg/day) pretreatment, VT also prolonged median survival (Fig 2A) by effectively suppressing SU-induced promotion of lung metastases, as seen by IVBI ($P < 0.05$, Fig 2D). Interestingly, while SU pretreatment promoted lung metastases (Fig 2D), it did not similarly promote lymphatic metastases (Fig 2H).

In another experiment (Fig 2E–H), IV injection of later-passage LM2-4luc cells into SCID mice led to extensive metastases in the lymphatics draining the tail vein, such that the main reason for sacrifice was hindlimb immobility. Here, of considerable interest, VT had no significant effect on survival rates (Fig 2E) or the progression of lymphatic metastases (Fig 2F–H)—thus implicating organ-specific or associated effects of VT on metastatic disease outcome.

**Colon cancer metastasis model: Vasculotide does not inhibit experimental HT29luc metastasis to the liver or lymphatics**

Since VT was effective at inhibiting hematogenously disseminated lung metastases, we asked whether it could also inhibit hematogenously disseminated liver metastases. Four to five weeks after IV injection of human colon cancer HT29luc cells (Hackl et al, 2013) into YFP-SCID mice, extensive experimental liver and lymphatic metastases could be observed (Fig 3A–D). Sacrificial endpoints were defined by immobility or ≥ 20% weight loss. No significant differences between PBS (control) vs. VT-treated mice were seen in terms of survival rates (Fig 3A), overall metastatic burden by whole-body IVBI (Fig 3B and C), or liver-specific metastases by necropsy (Fig 3D).

**Renal cancer metastasis model: Vasculotide does not inhibit experimental SN12luc metastasis to the lungs**

A predominance of experimental lung metastases also developed after the tail-vein injection of human renal cancer SN12-PM6-L1luc cells, referred to as ‘SN12luc’ hereafter (Jedzesko et al, 2015). SN12luc-derived lung metastases did not respond to VT treatment, whether given at the standard 250 ng/2d or a higher 400 ng/2d dose (Fig 3E–G), in contrast to the responsiveness of LM2-4luc-derived lung metastases to 250 ng/2d days VT treatment (Fig 2A–D). Since the same pulmonary endothelium was targeted in both cases, the variable in vivo efficacy of VT in curtailing lung metastases appeared to be dependent on the originating cancer cells.

**Endothelial cell-activating cytokines produced by Tie2+ tumor cells may influence the efficacy of Vasculotide in vitro**

One way by which the originating TCs could influence the in vivo efficacy of VT might be through their differential production of EC-activating cytokines. To investigate this possibility in vitro, we performed modified Boyden chamber assays where we stimulated ECs with tumor cell-conditioned media (TC-CM)—that is, supplement-reduced EC growth media containing all the cytokines naturally secreted by LM2-4luc, SN12luc, or HT29luc cells over 30 hours of hypoxic incubation (1% $O_2$).

In the presence of TC-CM stimulation, Ang1 or VT treatments reduced transendothelial macromolecular permeability ($P < 0.05$; Fig 4A) and TC migration ($P < 0.05$ for Ang1 and $P = 0.10$ for VT; Fig 4B and C). Interestingly, the degree of Ang1 or VT treatment efficacy varied depending on the type of TC-CM used (Fig 4B); for instance, VT treatment achieved greater inhibition of TC migration, on average, in the presence of LM2.4luc-conditioned media (~43%) compared to SN12luc-conditioned media (~23%). We asked whether this could be due to differences in cytokine composition between the three TC-CM types.

By ELISA or flow cytometric bead-based immunoassays, we quantified the media concentrations of three permeability-inducing
Figure 1. Vasculotide and Ang1 treatments counteract thrombin induction of trans-endothelium macromolecular permeability and tumor cell migration in vitro.

A–E Microvascular leak and tumor cell extravasation were modeled in vitro using modified Boyden chamber experiments where lung HMVECs (A–C) and dermal HMVECs (D–E) were grown to 100% confluence over 8-μm-pore insert membranes. ECs were first treated with Vasculotide (VT), PBS (vehicle/negative control), Ang1 (positive control), PEG-Cys (polyethylene glycol backbone), T7c (non-PEGylated CHHHRHSF peptides), or VT in the presence of 100-fold molar excess of T7c. The concentrations used, 10–20 ng/mL VT and 200–400 ng/mL Ang1, are estimated molar equivalents (0.71–1.43 nM). Thirty minutes later, ECs were stimulated with thrombin, 0.1% BSA (vehicle/negative control), or EDTA (positive control). Another 30 mins later, the amount of FITC-dextran diffusion into the lower chambers (A, D) provides a measure of endothelial permeability. CMTPX-labeled tumor cells (TCs) were then dispensed into inserts, and the amount of TC fluorescence emitting from the underside of insert membranes after 20 h (B–C) or 28 h [E] reflects the efficiency of trans-endothelial TC migration. Representative fluorescent images (10×) of membrane undersides are shown in (C), where DAPI-stained nuclei of ECs/TCs are shown in blue, ‘extravasated’ CMTPX-labeled TCs are shown in red, and the # of CMTPX+ pixels are shown numerically in red. Means ± SEM are shown (A,B,D,E). Three experiments (twice with HMVEC-LBl and once with HMVEC-DBl) were run with n = 3–5 inserts (independent biological replicates) per group and analyzed by two-sampled unpaired t-tests.
and/or pro-inflammatory cytokines, whose elevated levels in cancer patients often correlate with disease progression and poor prognosis: VEGF, IL-8, and IL-6 (Ferrara, 2005; Kut et al., 2007; Lippitz, 2013). Both VEGF and IL-8 can directly activate ECs, via VEGFR2 and CXCR1/CXCR2 receptors, respectively (Kim et al., 2001a; Le Guelte et al., 2011). In contrast, IL-6 primarily targets IL-6R+ leukocytes/TCs and only indirectly affects IL-6R+/ECs (Romano et al., 1997; Lo et al., 2011). We found that the media concentrations of VEGF and IL-8, but not IL-6, were considerably higher in LM2-4-conditioned media compared to other TC-CM types (Fig 4D)—suggesting that the contribution of LM2-4-derived VEGF/IL-8 to endothelial gap formation (Fig 4A) and TC diapedesis (Fig 4B) may be particularly amenable to counteraction by Ang1 or VT.

**Spontaneous breast cancer metastasis model: Vasculotide treatment does not suppress orthotopic primary tumor growth but inhibits metastasis to the lungs**

Orthotopic implantation experiments were also performed with the LM2-4luc cells, to further explore the effects of VT on primary tumor growth vs. spontaneous metastatic dissemination.
The first experiment involved VT and SU treatments in the presence of an unresected primary tumor (Fig 5). At 10 days post-implantation (DPI), when primary tumors reached an average size of ~175 mm³, mice were randomized into groups and chronic VT therapy was initiated. At 17 DPI, when tumors reached an average size (~400 mm³) where tumor debulking is typically required for long-term metastasis experiments, a 7-day anti-angiogenic SU treatment was initiated in lieu of surgical resection. SU effectively stabilized primary tumor growth (Fig 5A–C). In contrast, VT did not alter the growth kinetics of primary tumors, as measured by volume or bioluminescence (Fig 5A–C). This was consistent with *in vitro* observations from an MTS cell viability assay (Supplementary Fig S2), where VT had no direct cytotoxic effect on Tie2⁺ TCs and did not interfere with SU inhibition of Tie2⁺ ECs. Histological analysis of primary tumors showed that the extent of tumor cell viability (assessed by H&E staining; Fig 5D) and tumor hypoxia (assessed by IHC staining of CAIX, a target of the hypoxia-inducible transcription factor, HIF-1; Fig 5E) also remained unchanged after 22 days of VT treatment.

However, as detected by organ-specific *ex vivo* bioluminescent imaging (EVBI) upon sacrifice, there were trends of reduced incidences of metastatic involvement in the lungs, but no change in
Figure 4.  Endothelial cell-activating cytokines produced by Tie2+ tumor cells may influence the anti-permeability efficacy of Vasculotide and Ang1.

A, B In modified Boyden chamber experiments, confluent dermal HMVECs were stimulated with tumor cell-conditioned media (TC-CM)—that is, media conditioned by either LM2-4luc, SN12luc, or HT29luc tumor cells—and treated with 20 ng/mL Vasculotide vs. 400 ng/mL Ang1 (positive control) vs. PBS (negative control). Meta-analyses of treatment effects on dextran permeability (A) and tumor cell migration (B), where each datapoint represents the mean of an independent experiment, averaged over 2–3 biological replicates (inserts) per treatment group and normalized internally within that same experiment. Statistical significance of overall Ang1 or VT treatment effects was determined by paired t-tests, n = 11 (A) or 6 (B) experimental replicates.

C Representative fluorescent images (10×) of ‘extravasated’ CMTPX-labeled TCs (red) fixed on membrane undersides.

D LM2-4luc produced the highest levels of VEGF and IL-8, but lower levels of IL-6, compared to other TC types; ***P < 0.001 and *P < 0.05 (unpaired t-tests).
the lymph nodes, of the VT-treated groups, compared to their vehicle or SU monotherapy counterparts (Fig 5F). IHC-based size distribution analysis of metastatic nodules suggests that VT monotherapy delayed the seeding or progression of lung micrometastases, with larger nodules apparent only in the vehicle group and not the VT monotherapy group (P = 0.05-0.09; Fig 5G). Although a trend was observed by global EVBI analysis (Fig 5F), the anti-metastatic benefit of concurrent VT treatment was not detectable by regional IHC analysis (Fig 5G).

Combining Vasculotide with adjuvant anti-angiogenic therapy to target residual and micrometastatic disease after surgical resection of primary breast tumors

A second orthotopic implantation experiment involved surgical resection of primary LM2-4luc tumors (~400 mm³) at 17DPI (Fig 6) to model post-operative metastases arising from minimal residual disease (MRD). MRD includes the regrowth of incompletely resected mammary tumors, as well as circulating or disseminated tumor cells...
released from primary tumors prior to or at the time of surgical resection. VT was initiated 6 days prior to surgery and maintained chronically, while adjuvant SU (1-week or 3-week 60 mg/kg/day) treatments were initiated 1 day post-surgery (Fig 6A).

Two kinds of post-operative tumor burden, temporally and spatially distinct, were evident by bioluminescent imaging (Fig 6B):

(i) aggressive abdominal tumor burden—consisting of right inguinal mammary tumor regrowths, as well as metastatic expansions of pre-surgically invaded regional lymph nodes; and (ii) late-developing thoracic tumor burden—comprising lung metastases and axillary/brachial lymph node involvement. Within the critical 2.5 weeks post-surgery, during which we will contrast the effects of VT and SU treatment, locoregional proliferation/growth was the dominant driving force behind fast-expanding abdominal tumors, while in the thorax, TCs were still in the early stages of dissemination (extravasation, seeding and early colonization, etc.).

First, we observed that adjuvant SU treatment was more effective at inhibiting abdominal tumor growth (Fig 6D) than thoracic
metastatic dissemination (Fig 6C). We also noted the brevity of SU treatment efficacy—abdominal tumors in the ‘SU_3 wk’ group appeared to be stabilized so long as SU treatment was maintained (Fig 6B at 35DPI), but aggressively rebounded after cessation of SU treatment (Fig 6B at 49DPI). This transiency in tumor growth suppression by SU accounts for the longer median cessation of SU treatment (Fig 6B at 49DPI). This transiency in tumor growth suppression by SU accounts for the longer median cessation of SU treatment (Fig 6B at 49DPI). This transiency in tumor growth suppression by SU accounts for the longer median cessation of SU treatment (Fig 6B at 49DPI). This transiency in tumor growth suppression by SU accounts for the longer median cessation of SU treatment (Fig 6B at 49DPI). This transiency in tumor growth suppression by SU accounts for the longer median cessation of SU treatment (Fig 6B at 49DPI). This transiency in tumor growth suppression by SU accounts for the longer median cessation of SU treatment (Fig 6B at 49DPI). This transiency in tumor growth suppression by SU accounts for the longer median cessation of SU treatment (Fig 6B at 49DPI). This transiency in tumor growth suppression by SU accounts for the longer median cessation of SU treatment (Fig 6B at 49DPI).

In contrast, long-term VT monotherapy was more effective at inhibiting thoracic metastatic dissemination (Fig 6C, P = 0.04) than abdominal tumor growth (Fig 6D, P > 0.05). The survival advantage associated with VT monotherapy was not statistically significant despite an extension in median survival of 23 days (Fig 6A, \( P_{\text{log-rank}} = 0.42 \)). However, concurrent VT therapy conferred additive benefits—combining VT with 1-wk SU therapy, it lowered tumor bioluminescence to a similar extent as 3-wk SU monotherapy (Fig 6C and D).

**Vasculotide does not induce Tie2 phosphorylation in vitro or in vivo**

To follow-up on previous characterizations of VT as a Tie2-specific agonist, we stimulated primary human endothelial cells in vitro to directly assess the effects of VT on Tie2 phosphorylation status (Fig 7). Ang1, as our positive control, strongly and consistently activated Tie2 phosphorylation across the concentrations tested (15–400 ng/mL or 0.05–1.43 nM). VEGF, which is not a ligand of Tie2, was used as a negative control. Unexpectedly, VT treatment did not lead to any appreciable induction of Tie2 phosphorylation under any conditions tested in this study—whether given at concentrations of 5, 10, 20, 40, or 100 ng/mL (0.36–7.1 nM); at different timepoints (10, 15, or 45 min); with or without serum and supplement starvation; in the absence or presence of concurrent Ang1 stimulation; and in ECs of venous (HUVEC) or microvascular (HMVEC-Dbl) origin (Fig 7). Importantly, even though VT (10–20 ng/mL) and Ang1 (200–400 ng/mL) both functionally inhibited endothelial permeability in Boyden chamber assays where HMVEC-Dbl was used (Figs 1 and 4), the same concentration of VT (10 ng/mL) did not have similar activity as equimolar Ang1 (200 ng/mL or 0.7 nM) at the upstream level of Tie2 phosphorylation in the same dermal microvascular ECs (Fig 7F).

Since VT did not appear to be a direct Tie2 agonist within a reductionistic in vitro system of cultured ECs (Fig 7), we asked whether VT treatment may still indirectly lead to increased Tie2 phosphorylation within a physiological in vivo context. Tumor-free mice were treated for 1 week with either vehicle, VT (250 ng/2 days), SU (60 mg/kg/day), or VT+SU and then sacrificed 5 h after their last doses of treatment. Tissue homogenates derived from EC-rich organs (livers, lungs, kidneys) were subjected to Tie2 immunoprecipitation and then immunoblotted for phosphorylated Tie2 (Fig 8: ‘p-Tie2’) or total Tie2 (Fig 8: ‘Tie2’). Overall, in vivo VT treatment—whether as a monotherapy or when combined with SU—did not increase the ratio of phosphorylated Tie2 relative to total Tie2; in fact, lung-specific pTie2/Tie2 was significantly lower in the ‘VT’ group vs. ‘vehicle’ group (Fig 8).

**Vasculotide does not bind the extracellular domain of Tie2**

To better understand the difficulties we had in observing Tie2 activation by VT, we sought to characterize the binding affinity and kinetics of VT to Tie2. Purified human or mouse Tie2-Fc (i.e., Fc-conjugated extracellular domains of Tie2) were first coupled to protein A sepharose beads for pull-down experiments (Fig 9A and B) or immobilized on a Biacore CM5 sensor chip for surface plasmon resonance (SPR) experiments (Fig 9C and Supplementary Fig S3). In the pull-down assays, human and mouse Tie2-Fc effectively pulled down the majority of available recombinant human Ang1 (rhAng1), but did not pull down any appreciable levels of VT (Fig 9A and B). Likewise, SPR experiments—performed at physiological conditions of 37°C and pH 7.4—showed rhAng1, BowAng1, and COMP-Ang1 to be high-affinity binders (dissociation constants, \( K_d \), of 0.2–1.4 nM) to human and mouse Tie2-Fc (Fig 9C and Supplementary Fig S3B), but no binding to Tie2-Fc was detectable for VT, VEGF, PEG-Cys, or T7c (Fig 9C and Supplementary Fig S3).

**Discussion**

We report here five new findings relevant to therapeutic manipulation of the vasculature for cancer treatment, especially of metastatic disease. First, VT inhibits vascular permeability to the extravasation of tumor cells in vitro. Second, consistent with the aforementioned findings, VT inhibits the early stages of the metastatic process in vivo and thus may be potentially effective as an adjuvant therapy; in contrast, it does not inhibit tumor growth per se, for example, of established primary tumors. Third, the anti-metastatic effects of VT appear to be organ and tumor dependent, for example, inhibition of lung metastasis of breast cancer was observed but not of liver metastasis by colorectal cancer cells nor of lung metastasis by renal cell carcinoma cells. Fourth, these aforementioned effects appear to be independent of binding to or activation of Tie2 and thus may work through a different and thus potentially novel mechanism. Fifth, a potentially important ‘off-target’ effect of a commonly used anti-angiogenic TKI, sunitinib, was observed—inhbition of Tie2 phosphorylation.

This study addresses a gap in cancer therapy research, where efforts have largely focused on identifying drugs that limit the local malignant growth of tumors, resulting in a paucity of treatments that limit the distant metastatic spreading of cancer (Steeg, 2012). For instance, sunitinib (SU), an anti-angiogenic TKI that target the VEGF receptors (VEGFRs), repeatedly failed in multiple phase III clinical trials and preclinical models of advanced metastatic breast cancer, whereas, in contrast, it demonstrated potency in inhibiting angiogenesis-dependent growth of primary breast tumors in preclinical models (Abrams et al, 2003; Guerin et al, 2013). Recent preclinical studies suggest that many anti-angiogenic TKIs may inadvertently destabilize the normal microvasculature of distant host organs to facilitate TC extravasation and promote paradoxical pro-metastatic side effects, for example, by targeting pericytes (Ebos et al, 2009; Chung et al, 2012; Cooke et al, 2012; Weli et al, 2012).
With this report, we present a novel anti-metastatic therapy, where we seek to curtail metastatic dissemination—particularly, TC extravasation through the host microvasculature of distant organs—rather than the growth per se of secondary tumors.

Vasculotide (VT) was purported to be an Ang1-mimetic Tie2-specific agonist (David et al., 2011; Kumpers et al., 2011; Korpela et al., 2014). We found that VT was able to impair TC diapedesis in vitro by way of improving endothelial barrier integrity. In vivo, VT subdued the pro-metastatic effects of SU treatment, without interfering with the anti-angiogenic growth-inhibitory effects of SU on primary tumors, in mouse models of breast cancer metastasis using the LM2-4Luc human cancer cell line. However, an in-depth investigation into the putative mechanism of action of VT did not yield any evidence of direct binding to Tie2 or agonistic induction of Tie2 phosphorylation. We conclude that the vascular-stabilizing effects of VT do not occur through Tie2 agonism; alternative mechanism(s) of action have yet to be determined.

Meanwhile, there appeared to be organ-specific heterogeneity in the anti-metastatic efficacy of VT in vivo, possibly due to biological and structural differences in the type of endothelium being targeted. We observed that VT most effectively inhibited LM2-4Luc

![Figure 7.](#)
extravasation to the lung, but was ineffective against HT29LUC (a human colon cancer cell line) extravasation to the liver or LM2-4LUC/LM2-4Luc/HT29LUC infiltration of lymph nodes in mice. Moreover, not all cancer models with metastatic tropism to the lung uniformly benefited from VT treatment. In our study, lung metastases derived from LM2-4LUC (triple-negative breast cancer cells that secrete high levels of VEGF and IL-8), but not SN12LUC (VHL-wild-type renal cancer cells that secrete low levels of VEGF and IL-8), responded to VT treatment.

Vasculotide inhibits metastatic dissemination by targeting the established host vasculature

Using modified Boyden chamber experiments, we showed as a proof-of-concept that VT treatment could limit TC diapedesis across dermal or lung HMVECs that had been activated by various cancer-associated cytokines including thrombin, VEGF, and IL-8. The efficacy of VT in our in vitro dextran permeability assays can be ascribed to the ability of VT to prevent inter-EC gap formation (David et al., 2011). The efficacy of VT in our in vitro TC migration assays may additionally reflect an attenuation of EC surface adhesion molecules that can facilitate TC diapedesis—for example, VCAM-1 and ICAM-1 (Kumpers et al., 2011).

Given the lack of inhibitory effects by VT on the viability of proliferating ECs and TCs in vitro, it was not surprising that VT did not inhibit primary mammary tumor growth rates in vivo. Accordingly, the inhibition of secondary tumors in the lungs by VT also would not have occurred through anti-angiogenic targeting of sprouting ECs or cytotoxic targeting of proliferating TCs. Instead, we propose that VT delayed TC extravasation or metastatic seeding in...
the lungs in vivo mainly by stabilizing the barrier function of mature blood vessels in the lungs (e.g., by limiting permeability). The type of vascular endothelium traversed by TCs en route to their metastatic destination (Supplementary Fig S6) is likely to influence the efficacy of VT (Strell & Entschladen, 2008). The continuous non-fenestrated blood vessels of the pulmonary capillary beds were found to be most ‘targetable’ by VT. In comparison, VT treatment did not discernibly affect TC transit across the discontinuous fenestrated endothelium of the hepatic sinusoids or the loose cell–cell junctions of lymphatic capillaries.

Additionally, TC-derived permeability-inducing cytokines may also affect EC barrier-enhancing potential of VT treatment. In vitro, VT inhibition of TC migration across dermal HMVECs was greater in LM2-4Lac-conditioned media containing high levels of VEGF/IL-8 than in SN12Lac-conditioned media containing low levels of VEGF/IL-8. In vivo, VT effectively inhibited the progression of lung metastasis from LM2-4Lac cells but not from VHL-wild-type SN12Lac cells. An interesting possibility is that VEGF/IL-8-driven TC extravasation across lung HMVECs may be particularly amenable to VT counteraction. While VEGF and IL-8 are two of the most potent

![Figure 9](https://example.com/figure9.png)

**Figure 9.** Binding of Vasculotide vs. Ang1 variants to the extracellular domain of Tie2.

A–B  Pull-down assay. Input ligands—rhAng1 (A) and VT (B)—were incubated with either protein A sepharose beads only, bead-conjugated mouse Tie2-Fc (mTie2), or bead-conjugated human Tie2-Fc (hTie2). The ‘bound’ fraction shows the total amount pulled down by beads. The ‘unbound’ fraction is 7% of residual/free ligands remaining in the supernatant.

C  Quantitative kinetics analysis by SPR. All analytes were run at increasing concentrations of 0.625, 1.25, 2.5, 5, and 10 nM, at 37°C and pH 7.4. The positive controls—rhAng1, BowAng1, COMP-Ang1—all bound strongly to immobilized hTie2-Fc. There was no detectable binding of VT, or the negative control, rhVEGF, to immobilized hTie2-Fc.

Source data are available online for this figure.
and well-studied inducers of vascular permeability, there are potentially other factors that could also significantly modulate the efficacy of VT.

Is Vasculotide a Tie2 agonist or an Ang1 mimic?

By definition, a Tie2 agonist is a ligand that (a) directly binds to Tie2 receptors and (b) activates the tyrosine kinase activity and autophosphorylation of Tie2. In the assays performed for this study, we found no evidence of VT fulfilling either of these necessary requirements.

To our knowledge, the direct binding of the full VT to Tie2 has not been formally described or quantified. In their first paper, Van Slyke et al observed a faint Western blot signal when 20 nM of single-armed VT (i.e., one T7c peptide conjugated to a linear maleimide–PEG–biotin) was used to pull-down Tie2 from whole-cell lysates of EA.hy926 cells (Van Slyke et al., 2009). In contrast, the direct binding of tetravalent VT—whether avidin-conjugated (Van Slyke et al., 2009) or PEG-conjugated (David et al., 2011; Kumpers et al., 2011; Korpela et al., 2014)—to purified Tie2 has not been experimentally confirmed to our knowledge. The two direct binding assays used in this study, namely a pull-down assay and SPR analysis using purified Tie2-Fc, both revealed no detectable or quantifiable binding of PEGylated VT to the extracellular domain of Tie2.

Also unexpected was the undetectable binding of T7c peptides to Tie2-Fc by SPR. The main focus of the original paper that discovered T7 (Tournaye et al., 2004) was actually on a different peptide called ‘T4’ (NILMAAS) and its potential use as a Tie2 inhibitor/antagonist. When they initially screened phage-displayed heptapeptides by ELISA for Tie2-binding potential, T4 and T7 were among four candidates selected for further study. These candidate peptides, in their synthetic free form, were then subjected to competition assays by ELISA and SPR to show that T4 was able to competitively inhibit Ang1 or Ang2 binding to Tie2 ($K_i \sim 0.3$ mM), while T7 was not (tested up to 1 mM). In essence, these competition assays confirmed Tie2 binding for T4, but not for T7.

Our repeated investigations into the induction of Tie2 phosphorylation in vitro (analyzing primary venous and microvascular ECs treated directly with VT) and in vivo (analyzing lung, kidney, and liver tissue from mice treated with VT) also did not confirm the anticipated agonistic activity of PEGylated VT on Tie2. Of the three published papers on PEGylated VT, only the most recent study showed direct testing of VT on ECs in vitro, albeit on hTERT-immortalized ECs (Korpela et al., 2014). To show Tie2 phosphorylation or dependency, the older studies relied on in vivo systems, where indirect dependency and sample heterogeneity (e.g., variability in EC content) are inherently more difficult to exclude or account for, particularly when crucial controls are absent.

Altogether, Tie2 agonism is unlikely to be the mechanism of VT’s vascular-stabilizing effects. Since many other signaling pathways (e.g., VEGF, thrombin, IL-8, Ca$^{2+}$) crosstalk and converge to govern the same biological responses that are regulated by Ang1-Tie2 signaling (Le Guelte et al., 2011; Koh, 2013), it would be premature to categorize VT as an Ang1 mimic based on similarity in downstream function alone.

Further studies will be needed to determine whether VT has any other molecular binding partners, and if so, the binding affinity, kinetics, specificity, and biological significance of such interactions. Studies are in progress to elucidate possible mechanisms by which VT can cause the functional effects we have observed.

Does sunitinib inhibit tumor growth, by targeting the developing tumor neovasculature, while potentially exacerbating tumor spread?

Many anti-angiogenic TKI therapies have underperformed clinically for patients with advanced metastatic breast cancer (MBC)—especially with sunitinib (SU) treatment, which when given as a monotherapy or combined with conventional chemotherapies has repeatedly failed in multiple phase III clinical trials (Ebos & Kerbel, 2011; Mackey et al., 2012). These clinical results were recently replicated in a preclinical model of postsurgical advanced MBC (Guerin et al., 2013). As discussed previously (Guerin et al., 2013), primary and secondary tumors may have divergent responses to anti-angiogenic therapies due to differences in their relative dependency on angiogenesis.

Another possible explanation for the apparent insensitivity of MBC to TKI treatments is that the growth of secondary tumors post-metastatic colonization of the lung/liver may still be susceptible to the intended anti-angiogenic inhibition, but that these growth-inhibitory benefits may be diluted by concurrent unintended drug effects that promote tumor spread. This appears to be the case in our resected orthotopic LM2.4Luc experiment, where adjuvant SU treatment transiently suppressed the locoregional growth of residual abdominal tumors, but was ineffective against the distal dissemination of thoracic tumors. Counterproductive dissemination-promoting side effects may explain the clinical observations from a phase II study of advanced MBC where response rates to sunitinib differed between patients with locoregionally growing superficial metastatic disease (20%) and patients with distally disseminated visceral metastatic disease (9%) (Yardley et al., 2012).

An interesting and unexpected finding from our own study where Tie2 dephosphorylation in the host vasculature may be yet another unintended consequence of TKI treatments which could promote metastatic seeding. This adds to other mechanisms previously proposed: therapy-induced tumor hypoxia could activate HIF-1 and HGF/Met pathways that increase tumor invasiveness and TC intravasation (Paez-Ribes et al., 2009; Cooke et al., 2012); host responses that upregulate circulating pro-angiogenic factors (Ebos et al., 2007) could enhance vascular permeability and TC diapedesis; and the destabilization of inter-EC junctions and pericytes in host organs could promote TC arrest or extravasation (Chung et al., 2012; Welti et al., 2012).

Also, organ specificity in relation to the pro-metastatic potential of SU was noted in this study—SU pretreatment preferentially accelerated experimental lung metastasis, but not lymphatic metastasis, from IV-injected breast cancer cells. This finding adds to a prior report where SU treatment of pancreatic neuroendocrine tumors increased metastasis to the liver but not the lymph nodes (LNs) (Paez-Ribes et al., 2009). There are at least two plausible explanations for this differential impact of SU on hematogenous metastasis (to lung/liver) vs. lymphogenous metastasis (to LNs). Paez-Ribes et al. (2009) hypothesized that the concomitant disruption of lymphatic EC signaling via VEGF-R3 inhibition by SU, a broad-spectrum TKI, may have prevented its general pro-metastatic potential from actualizing through the lymphogenous route. Moreover,
PDGFR inhibition and pericyte depletion by SU had been implicated in its augmentation of lung metastasis (Cooke et al., 2012; Welti et al., 2012); since lymphatic vessels are not covered by pericytes, one would not expect a similar enhancement of LN metastasis through this mechanism.

**Complementary anti-metastatic strategies: combining host-targeting vascular stabilization with tumor-targeting anti-angiogenic therapy**

In summary, our results suggest that the mechanisms of action and therapeutic effects of sunitinib (SU) treatment were distinct from—and perhaps complementary to—that of VT treatment. Anti-angiogenic SU therapy inhibits tumor growth, at least in part by targeting the tumor neovasculature to suppress the ‘angiogenic switch’ (Bagri et al., 2010), but may inadvertently promote metastatic dissemination. EC-stabilizing VT therapy targets the normal vasculature of host organs, especially in the lungs, to prevent metastatic extravasation, while having no effect on tumor growth per se. As such, a potentially promising application for anti-metastatic VT therapy to consider would be in the post-surgical micrometastatic disease setting, where VT could reduce the potential disadvantages associated with discontinuous adjuvant anti-angiogenic sunitinib therapy, or surgery-induced spreading of residual tumor cells (Goldfarb & Ben-Eliyahu, 2006).

**Materials and Methods**

**In vitro modified Boyden chamber experiments**

Endothelial cells (ECs) were grown on cell culture inserts with uncoated 8-μm-pore PET filter membranes to 100% confluency. See Supplementary Fig S4 for detailed timelines. Experiments began with a media change, introducing EC-stabilizing treatments (VT (Bachem); or rhAng1 (R&D Systems)) and EC destabilizing stimuli (thrombin; EDTA; or tumor cell (TC)-conditioned media) into both upper and lower chambers. Permeability assays: A total of 100 μg of FITC-dextran (Sigma FD-20S) was dispensed into the upper chamber media. After 30 min of thrombin stimulation or 4-6 h of TC-CM stimulation, FITC fluorescence (ex/em = 490/520 nm) was measured from a 50 μl sample of the lower chamber media. Tumor cell extravasation assays: A total of 4 × 10^6 TCs freshly labeled with CellTracker™ Red CMPTX (Invitrogen C34552; ex/em = 577/602 nm) were seeded into each insert. Where EDTA was used in the permeability assay, the assay media was changed to remove EDTA before introducing TCs. After a 15- to 30-h incubation, the ECs and non-migrated TCs above insert membranes were removed with a cotton swab, whereas migrated/‘extravasated’ TCs and ECs below insert membranes were fixed in 4% PFA. Fixed membranes were mounted onto slides with DAPI stain. CMPTX fluorescence from migrated TCs was then quantified from 10× microscopy images using a MATLAB (MathWorks, Natick, MA, USA) script; five images (technical replicates) were analyzed per insert.

**Animal experiments**

In vivo experiments were performed in strict accordance with protocols approved by the Sunnybrook Research Institute Animal Care Committee, accredited by the Canadian Council of Animal Care. All surgical, imaging, and euthanasia procedures were performed under inhaled isoflurane anesthesia. Experimental metastasis models involved injections of 10^6 tumor cells, suspended in 200 μl serum-free DMEM, into the mouse tail vein. LM2-4luc implantations were performed on 6-8-week-old female CB-17 SCID mice (Charles River Canada). HT29luc implantations were performed on 19- to 20-week-old male and 11- to 12-week-old female YFP-SCID mice (bred in-house). SN122luc implantations were performed on 9-week-old male YFP-SCID mice. Spontaneous metastasis models involved orthotopic implantations of 2 × 10^6 LM2-4luc cells/50 μl serum-free DMEM in the right inguinal mammary fat pads of 6-week-old female CB-17 SCID mice.

**Drug preparation and in vivo dosing**

Sunitinib malate (SU), from Pfizer or LC Laboratories, and its vehicle were formulated as described before (Ebos et al., 2009). SU was administered by oral gavage (PO), once daily, at a dose of 60 mg/kg mouse weight. Vasculotide (VT): VT synthesized at the Sunnybrook Research Institute (Toronto, ON, Canada) was used in experiments described in Fig 2(A-D) and Supplementary Fig S5(A and B). VT synthesized by American Peptide (Sunnyvale, CA, USA) was used in experiments described in Fig 5. VT synthesized by Bachem UK Ltd was used in experiments described in Figs 1, 2(E-H), 3, 4, 6–9, Supplementary Figs S2, and S5(C and D). Lyophilized product was reconstituted in PBS to 500 μg/500 μl stock aliquots and stored at

**The paper explained**

**Problem**

‘Anti-angiogenic cancer therapies’ seek to inhibit the sprouting growth of new blood vessels that feed growing/expanding tumors. Approved anti-angiogenic drugs that target the VEGF pathway have had modest clinical success in many types of advanced metastatic cancer, and early clinical trial results in the post-surgical adjuvant treatment setting have been disappointing. Preclinical studies have uncovered mechanisms whereby treatments such as VEGF pathway inhibition and surgical resections can counterintuitively aggravate metastatic dissemination from treated tumors, despite obvious utility of the same treatments in debulking or stabilizing localized tumor growth. Vasculotide (VT)—a synthetic compound presumed to be a specific activator of the Tie2 signaling pathway—was reported to have vascular-stabilizing effects. In this study, we tested whether VT can reduce metastasis by limiting the permeability of systemic blood vessels to tumor cell extravasation.

**Results**

Unexpectedly, extensive mechanistic analyses revealed no evidence of direct binding to Tie2, nor direct activation of Tie2 phosphorylation. Nonetheless, VT reduced trans-endothelial permeability and tumor cell migration in cell culture. In a mouse model of breast cancer, we found that VT did not affect primary tumor growth but delayed metastatic spread to the lungs.

**Impact**

This study presents a novel combinatorial approach to anti-metastatic therapy, whereby a VEGF receptor-targeting anti-angiogenic drug (sunitinib) that inhibits local tumor growth may be complemented by a Tie2-independent vascular-stabilizing agent (VT) that inhibits distant metastatic spread.
–80°C. As needed, stock aliquots were diluted with PBS to a 250 ng/50 µl working concentration and stored in 0.4–1 ml aliquots at –80°C. VT (at a standard dose of 250 ng/mouse) or its vehicle (PBS) was administered to mice by intraperitoneal (IP) injections every other day.

Statistical analysis

GraphPad Prism software (San Diego, CA, USA) and Microsoft Excel were used for statistical analysis. Kaplan–Meier survival curves were compared by either the log-rank test (\(P_{\text{log-rank}}\)) when evaluating chronic therapies or the Gehan–Breslow–Wilcoxon test (\(P_{\text{GWW}}\)) when evaluating the long-term effects of transient/terminated therapies. Bioluminescent fluxes were log-transformed prior to comparison by Mann–Whitney tests (two-sample) or Kruskal–Wallis tests (with Dunn’s post-tests on planned comparisons) for non-Gaussian distributions, and \(t\) tests (two-sample) or one-way ANOVA (with Bonferroni’s post-tests on planned comparisons) for Gaussian distributions. For all other results, see figure legends for specific statistical tests used.

Supplementary information for this article is available online:
http://embomolmed.embopress.org

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Author contributions

RSK supervised the entire study; JG supervised the SPR experiments. All authors contributed to the design and interpretation of experiments. FTHW and CRL performed the in vivo experiments. FTHW performed the histological and Boyden chamber assays. FTHW and EB performed the Tie2 phosphorylation and Tie2-binding pull-down experiments. EB performed the COMP-Ang1 purification. AP performed the Tie2-binding SPR experiments. FTHW and RSK wrote the manuscript. All authors critically reviewed this manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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