



In vitro inhibition of cancer angiogenesis and migration by a nanobody that targets the orphan receptor Tie1

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Abstract

The human signaling molecules Tie1 and Tie2 receptor tyrosine kinases (RTKs) play important pathophysiological roles in many diseases, including different cancers. The activity of Tie1 is mediated mainly through the downstream angiopoietin-1 (Ang1)-dependent activation of Tie2, rendering both Tie 1 and the Tie1/Tie2/Ang1 axis attractive putative targets for therapeutic intervention. However, the development of inhibitors that target Tie1 and an understanding of their effect on Tie2 and on the Tie1/Tie2/Ang1 axis remain unfulfilled tasks, due, largely, to the facts that Tie1 is an orphan receptor and is difficult to produce and use in the quantities required for immune antibody library screens. In a search for a selective inhibitor of this orphan receptor, we sought to exploit the advantages (e.g., small size that allows binding to hidden epitopes) of non-immune nanobodies and to simultaneously overcome their limitations (i.e., low expression and stability). We thus performed expression, stability, and affinity screens of yeast-surface-displayed naïve and predesigned synthetic (non-immune) nanobody libraries against the Tie1 extracellular domain. The screens yielded a nanobody with high expression and good affinity and specificity for Tie1, thereby yielding preferential binding for Tie1 over Tie2. The stability, selectivity, potency, and therapeutic potential of this synthetic nanobody were profiled using in vitro and cell-based assays. The nanobody triggered Tie1-dependent inhibition of RTK (Tie2, Akt, and Fak) phosphorylation and angiogenesis in endothelial cells, as well as suppression of human glioblastoma cell viability and migration. This study opens the way to developing nanobodies as therapeutics for different cancers associated with Tie1 activation.

Keywords Nanobody · Tie1 · Protein engineering · Receptor tyrosine kinase · Angiogenesis · Cell migration

Abbreviations

RTKs	Receptor tyrosine kinases
Ang1	Angiopoietin-1
Fak	Focal adhesion kinase
PI3K	Phosphoinositide 3-kinase
Akt	Protein kinase B
LECT2	Leukocyte cell-derived chemotaxin 2
CDRs	Complementarity-determining regions

PDB	Protein data bank
FACS	Fluorescence activated cell sorting

Introduction

Orphan receptors, i.e., receptors whose natural ligands and biological functions are yet to be elucidated, present challenging targets for drug discovery, as they appear to be associated with a variety of diseases, although the particular ligands remain elusive. The current ‘lack’ of a natural ligand for the orphan receptor Tie1, a receptor tyrosine kinase (RTK), is undoubtedly complicating research on its function [1]. However, it is known that Tie1 and its family member Tie2, which are expressed in endothelial cells [2, 3], are key regulators of normal blood and lymphatic vessel development and also of pathological processes, including tumor angiogenesis, progression and metastasis, atherosclerosis, and vascular leakage [4, 5]. More specifically, it is known that Tie1, together with Tie2 and its natural ligand

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angiopoietin-1 (Ang1), is essential for normal blood vessel maturation [6–8], and that the expression of Tie1 is increased in tissue angiogenesis and oncogenic processes. Conversely, deletion of Tie1, and also of Tie2, results in reduced angiogenesis and tumor growth, for example, in implanted Lewis lung carcinoma (LLC), B16F10 melanoma (B16), or EL4 leukemia/lymphoma (EL4) cells [5, 9–11]. Importantly, it was shown that when Tie1 located on endothelial cell membrane complexes with the Tie2–Ang1 receptor–ligand pair, the complex became concentrated at the cell–cell contact interface [12–14], where Ang1 activated (although did not bind directly to) Tie1 [5, 12, 15]. Similarly, it was shown in mouse endothelial cells that in vivo Tie1 deletion reduces Ang1-induced phosphorylation of Tie2, indicating that Tie1 affected the process of Ang1-induced Tie2 activation and the downstream focal adhesion kinase (Fak)/phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway that led to tumor growth and vascularization [9, 16].

While the Ang1–Tie2 axis has been well researched, especially with regard to endothelial and cancer cell growth, migration, and survival [17–19], the signaling pathways and cellular functions regulated specifically by Tie1 are not completely understood, primarily due to the lack of suitable biochemical tools. The lack of progress in research on Tie1 would appear to be somewhat surprising in light of the close homology exhibited by Tie1 and Tie2 [5], with the amino acid sequences of their cytoplasmic kinase domains showing about 80% identity, although their extracellular domains are more diverse, sharing 33% identity and 49% similarity in sequence [20–23]. Thus, efforts are underway to pinpoint natural proteins and protein variants that specifically target the extracellular domain of Tie1 [12, 24, 25].

A possible way forward to revealing the signaling pathways and cellular functions regulated by Tie1 was thought to lie in the development of targeting antibodies; for example, a Tie1-targeted function-blocking antibody, designated AB-Tie1-39, inhibited Tie1 and thereby led to a decrease in Tie2-mediated Akt phosphorylation [24]. This research approach was, however, impeded by the well-known limitations of antibodies (despite their significant clinical success), including: their inability to specifically incorporate chemical modalities, their potential undesired effector functions [25, 26], the high cost of their recombinant production in mammalian cells [26], and the considerable intellectual property barriers to their development [27]. An answer to these limitations may lie in nanobodies, single-domain antibodies obtained from camelids (llamas, camels, alpacas, or their relatives) [28]. The inherent advantages of nanobodies include their small size (~ 15 kDa, 4 nm long and 2.5 nm wide), their high solubility, stability, specificity, affinity, their thermal and chemical resistance, and the ease of cloning them. In addition, nanobodies have a low immunogenic profile, which

makes them suitable for human use. Among the above properties of nanobodies, their small size is particularly important, because it facilitates their use for targeting antigens with small or inaccessible-binding pockets [29]. The interaction of nanobodies with such antigens is facilitated by the three complementarity-determining regions (CDRs) CDR1–3 and their corresponding framework residues. Of note, CDR3 is the main contributor to antigen specificity, whereas CDR1 and CDR2 are responsible for binding strength [30, 31].

In research and in therapeutic applications, nanobodies can be used either as antagonists (i.e., to compete for binding of effector molecules) or to create conformational (e.g., allosteric) changes in their target receptors that prevent their recognition by their natural ligands [29]. Therefore, the use of nanobodies rather than classical antibodies may provide the means to overcome some of the current challenges in the development of drugs targeting orphan receptors. However, current methods for generating nanobody-based compounds remain slow, expensive, and often unreliable. These problems derive primarily from the method of nanobody preparation, which includes the drawn-out and costly step of immunization of a camelid. To address these problems and to open the way for nanobody development, the previous efforts have combined yeast (or phage) display affinity screens with synthetic (non-immune) libraries [32]. However, it still remains particularly challenging to identify, in synthetic libraries, nanobodies that exhibit the desired combination of stability and high expression [33, 34]. It is against this background that we sought to develop both a nanobody that would bind strongly to Tie1 and a protocol for the inexpensive and easy production of such a nanobody. Our protocol was based on randomly mutating the CDR regions of a predesigned synthetic nanobody library, which was screened using a cell-based selection scheme enabled by fluorescence activated cell sorting (FACS). This library was previously obtained using an alignment of structurally characterized nanobodies from the Protein Data Bank (PDB). The methodology enabled us to identify a stable nanobody that binds strongly to Tie1 and also binds to Tie2 but less preferentially than to Tie1. This nanobody, which we designated NB19, was found to bind the recombinant human Tie1 extracellular domain, designated here as Tie1–ECD, with a K_D in the subnanomolar range and, importantly, to show selectivity for Tie1 over Tie2 in vitro. We also demonstrated that NB19 significantly inhibits Tie1 and Tie2 phosphorylation in a dose-dependent manner and thus acts as functional antagonist of Tie1, leading to reduced angiogenesis and inhibition of cancer viability and migration. By exploiting the ability of NB19 to bind both Tie1 and Tie2, but with preference for Tie1, we were able to explore and dissect out the specific roles of each of these two receptors in angiogenesis and cancer growth and migration.

Materials and methods

Generation and purification of the anti-Tie1 nanobody

The protocol for nanobody generation was adapted from Shlamkovich et al. [35]. The selected nanobody (i.e., NB19) was purified using periplasmic extraction, followed by Ni-NTA affinity chromatography and size-exclusion chromatography (SEC), as described in detail in the supplementary information (SI) section.

NB19-binding analysis using surface plasmon resonance (SPR)

The binding interactions between NB19 and Tie1-ECD were analyzed (Proteomics Unit, NIBN, BGU) in real time by surface plasmon resonance (SPR) on a ProteOn XPR36 instrument (Bio-Rad, Hercules, CA, USA). The ProteOn GLC sensor chip was initialized in air, and PBST (PBS, 0.005% Tween) buffer was flushed through the instrument prior to binding experiments. The purified NB19 protein was immobilized on the surface of the GLC sensor chip using the amine coupling reagents *N*-hydroxysuccinimide (0.1 M; sulfo-NHS) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (0.4 M; EDC; Bio-Rad), as follows. NB19 (10 µg) in 10 mM sodium acetate, pH 4.0, was allowed to flow over an activated GLC sensor chip channel surface at a flow rate of 30 µL/min, until the target immobilization level was reached. Bovine serum albumin (BSA), 3 µg in 10 mM sodium acetate, pH 4.5, was then allowed to flow over the activated surface of a control GLC sensor chip channel at a flow rate of 30 µL/min until the NB19 ligand immobilization level was reached. After NB19 immobilization, the chip surface was treated with 1 M ethanolamine HCl at pH 8.5 to deactivate excess reactive esters. All binding experiments were performed at 25 °C in a degassed binding buffer (PBS, pH 7.4). Recombinant human Tie1-ECD (R&D Systems, Minneapolis, MN, USA) and recombinant human Tie2 extracellular domain (Tie2-ECD, R&D Systems) at a range of concentrations (10–0.625 nM and 50–6.25 nM, respectively) were allowed to flow over the surface-immobilized NB19 at a flow rate of 30 µL/min for 60 min for monitoring Tie1-ECD–NB19 and Tie2-ECD–NB19-binding interactions. After complex dissociation, which was monitored for 60 min, a regeneration step with 50 mM NaOH at a flow rate of 100 µL/min was performed. The analyte sensorgram run was normalized by subtracting the BSA-immobilized channel and the zero-analyte concentration runs. The binding constant (K_D) was determined from the

sensorgrams of the equilibrium-binding phase. Binding kinetics of NB19 were analyzed by fitting to a Langmuir model.

Cell culture

Human embryonic kidney 293 cells (HEK293; ATCC, Manassas, VA, USA) were cultured in complete growth medium composed of DMEM High Glucose (Biological Industries, Kibbutz Beit-Haemek, Israel) supplemented with 10% fetal bovine serum (FBS, Thermo-Fisher Scientific, Waltham, MA, USA), 1% L-glutamine (Biological Industries), and 1% penicillin/streptomycin (Biological Industries) under 5% CO₂ at 37 °C. HEK293 cells, which lack endogenous Tie receptors, were transiently transfected with full-length HA-tagged Tie1 (pCMV3-TIE1-HA refseq: NM_005424.2) and full-length myc-tagged Tie2 (pCMV3-C-Myc redseq: NM_000459.3). When cells reached 80–90% confluence in 6-well plates, transfections were performed using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. Human telomerase-immortalized microvascular endothelium (TIME) cells (ATCC), which endogenously express Tie1 and Tie2, were cultured under 5% CO₂ at 37 °C in Vascular Cell Basal Medium (ATCC) supplemented with 10% FBS and Microvascular Endothelial Cell Growth Kit-VEGF (ATCC) according to the manufacturer's instructions. Human glioblastoma (U87-MG) cells were grown under 5% CO₂ at 37 °C in minimum essential medium supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin.

Cell-binding assay using flow cytometry

Expression of Tie1 and Tie2 and binding of purified NB19 to receptor-expressing cells were analyzed by flow cytometry using TIME or U87-MG cells endogenously expressing Tie1 and Tie2 and HEK293 cells overexpressing Tie1, Tie2, or both receptors. To determine the expression levels of Tie1 and Tie2 in HEK293 cells following transient transfection, cells were centrifuged and resuspended in 100 µL of PBSA with 1:100 allophycocyanin (APC)-labeled anti-human Tie2 antibody (BioLegend) or phycoerythrin (PE)-labeled anti-human Tie1 antibody (R&D Systems), incubated at 4 °C for 30 min, and washed three times with 100 µL of phosphate-buffered saline (PBS) containing 1% bovine serum albumin (PBSA) before flow cytometry analysis. Unstained cells were used to determine the background signal. For binding assays, Tie1- and Tie2-expressing TIME (or U87-MG) cells and HEK293 cells transiently transfected with Tie1, Tie2 or both receptors were incubated with NB19 at different concentrations in a total volume of 200 µL of PBSA at 4 °C for 1 h with gentle agitation. Thereafter, the cell suspensions were centrifuged at 150g at 4 °C for 5 min and

washed three times with 100 μ L of PBSA. Cells were then resuspended in 100 μ L of PBSA containing a 1:200 dilution of APC-conjugated anti-FLAG antibody (BioLegend) for the detection of NB19 bound to cells. After 30 min on ice, cells were washed twice in PBSA and analyzed by flow cytometry with a BD FACSCanto™ II Flow Cytometry System (BD Biosciences, San Jose, CA, USA). Mean fluorescence levels of the background were subtracted, and the data were analyzed using FlowJo (Tree Star Inc., OR, USA) analysis software. Binding analysis was performed using one- and two-site specific binding models in GraphPad software.

Immunofluorescence microscopy

HEK293 cells were seeded on sterile μ -Slide 8 Well (ibidi, Gräfelfing, Germany) and grown to confluence before being transfected using Lipofectamine 2000 (Invitrogen) with plasmids encoding either full-length HA-tagged Tie1 (pCMV3-TIE1-HA; refseq: NM_005424.2) or full-length myc-tagged Tie2 (pCMV3-C-Myc; redseq: NM_000459.3). Untransfected cells were used as the negative control. Twenty-four hours post-transfection, cells were washed with PBS and then incubated for 30 min at 4 °C with 100 nM NB19. Cells were then washed with PBS, fixed for 15 min in 4% paraformaldehyde, and permeabilized for 10 min with 0.5% Triton X-100 (Bio-Lab Ltd, Jerusalem, Israel) in PBS. Cells were blocked for 30 min with gentle shaking at room temperature in 5% BSA in PBS, followed by two washes with PBS. Cells were then incubated in 5% BSA/PBS for 1 h at 4 °C with FITC anti-human Tie2 (BioLegend) or PE-labeled anti-human Tie1 (BioLegend), and APC-conjugated anti-FLAG antibody (BioLegend) for the detection of NB19 colocalization with Tie1 and Tie2. Confocal images were captured using a 20 $\times$ Zeiss Plan-Apochromat dry, 0.8 NA, DIC objective on a laser scanning confocal microscope (Zeiss LSM880 inverted microscope, Jena, Germany) with Zeiss Zen black v2.3 software. The excitation lasers used were FITC (488 nm), PE (561 nm), and APC (633 nm), and the corresponding emission detection wavelengths were FITC (481–541 nm), PE (552–615 nm), and APC (634–755 nm).

Image analysis

Colocalization was calculated using Zen Blue v3.5 software. The background level was determined using the “Costes method” [36] for each image individually.

Inhibition of Tie1 by siRNA

For small interfering RNA (siRNA)-mediated knockdown of Tie1, TIME cells were plated at 80% confluency 1 day before transfection and then transfected with Tie1 siRNA

using the TriFECTa DsiRNA kit (hs.Ri.TIE1.13, IDT, San-Jose, CA, USA) with the Lipofectamine 3000 reagent (Invitrogen) according to the manufacturer’s instructions.

Western blot detection of Tie1, Tie2, Akt, and Fak phosphorylation

Confluent TIME cells were cultured on 12-well plates in growth-factor depleted Vascular Cell Basal Medium (ATCC) supplemented with 0.5% FBS for 12 h at 37 °C, under 5% CO₂. Cells were then washed with PBS, and the medium was exchanged with fresh Vascular Cell Basal Medium depleted of growth factors and serum (ATCC). Following treatment with 1 mM sodium orthovanadate (Na₃VO₄; Sigma) for 15 min, the cells were co-incubated for 15 min (for Tie1 and Tie2 analysis), or for 30 min (for Fak and Akt analysis), at 37 °C with either 500 ng/mL human recombinant Ang1 (R&D systems) as the positive control, or 30 nM (or 300 nM) NB19 combined with 500 ng/mL Ang1. Non-stimulated (in the absence of Ang1) cells served as the negative control. Cells were then washed twice with PBS containing 1 mM Na₃VO₄ and lysed in ice-cold lysis buffer [20 mM HEPES, pH 7.4, 150 mM NaCl, 1% TritonX-100, 1 mM Na₃VO₄, and 1 $\times$ complete protease inhibitor cocktail tablet (Roche, USA)]. Cells were scraped from the culture plate wells, and the lysates were clarified by centrifugation (13,000 rpm for 30 min at 4 °C). Protein concentration was measured by the bicinchoninic acid (BCA) assay (Thermo-Fisher Scientific), and equivalent amounts of each lysate sample were analyzed by 10% SDS-PAGE transferred to PVDF blotting membranes (Bio-Rad). Blots were blocked (5% BSA, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) for 1 h at room temperature and then probed, following an overnight incubation at 4 °C, with the following antibodies, all at a 1:1000 dilution: phospho-Tie1 (Tyr1117) specific rabbit polyclonal antibody (Invitrogen), anti-Tie1-specific rabbit monoclonal antibody (Cell Signaling Technology, Danvers, MA, USA), phospho-Tie2 (Y992) specific rabbit polyclonal antibody (R&D Systems), anti-Tie2-specific rabbit monoclonal antibody (Cell Signaling Technology), anti-phospho-Akt-specific antibody (Cell Signaling Technology), anti-Akt-specific antibody (Cell Signaling Technology), anti-phospho-Fak-specific (Tyr397) antibody (Cell Signaling Technology), or anti-Fak-specific antibody (Cell Signaling Technology). PVDF membranes were washed three times with TBST (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) and probed with a 1:1000 dilution of HRP-linked anti-rabbit antibodies (Cell Signaling Technology) for 1 h at room temperature. Membranes were washed three times with TBST and then visualized and quantified using chemiluminescence (ECL, Biological Industries) and ImageJ software, respectively. The intensities of the phospho-Tie1, phospho-Tie2, phospho-Akt, and

phospho-Fak bands were adjusted for the expression of total Tie1, Tie2, Akt, and FAK, respectively, for each experiment. Blots were stripped and re-probed with a 1:1000 dilution of anti- β -actin antibody (Cell Signaling Technology) followed by an HRP-conjugated anti-mouse antibody (at 1:1000 dilution, Cell Signaling Technology) for further normalization. Each experimental condition was repeated in triplicate. Band intensities of the phosphorylated proteins (Tie1, Tie2, Akt, and Fak, as measured by ImageJ software) were normalized to the respective total protein levels, and this value was subsequently normalized to the total amount of β -actin for each sample.

Capillary-like tube formation assay

Serum-reduced Matrigel (10 mg/ml; BD Biosciences) was thawed overnight at 4 °C, and 150 μ L were added to each well of a μ -Slide 8 Well (ibidi, Gräfelfing, Germany) and allowed to solidify for 1 h at 37 °C. In each well, the Matrigel was then incubated with 1×10^5 TIME cells treated with either 500 ng/mL Ang1 or with a combination of 500 ng/mL Ang1 and 30 nM (or 300 nM) NB19; untreated cells were used as the negative control. Cells were incubated for 18 h at 37 °C, under 5% CO₂, and then washed twice in Hanks' balanced salt solution (Sigma-Aldrich). Capillary tube formation was observed on confocal images captured using the Zeiss Plan-Apochromat laser scanning confocal microscope described above and analyzed with Zeiss software. Tube structures were analyzed for the number of junctions generated, and the total tube length was quantified by the analysis of digitized images of the capillary-like structures using ImageJ software and the Angiogenesis Analyzer plugin.

Cell viability assay

The effects of NB19 on the growth and survival of TIME or U87-MG cells were assessed by an XTT assay (2,3-bis [2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide inner salt assay; Biological Industries). TIME or U87-MG cells were seeded (7500 cells per well) on a human vitronectin-coated 96-well microplate (R&D systems) and incubated in growth medium for 24 h at 37 °C, under 5% CO₂. The cell growth medium was then replaced with fresh vascular cell basal medium (ATCC) or minimum essential medium for TIME or U87-MG cells, respectively. In each case, the medium was supplemented with 2% FBS and growth-factor supplements, and cells were incubated with either 500 ng/mL Ang1 or with a combination of 500 ng/mL Ang1 and 30 nM (or 300 nM) NB19 for 24 h at 37 °C, under 5% CO₂. Viable cells from each condition were measured by XTT at UV 450 nm, as described in the manufacturer's protocol. The UV readings of the cell-only (untreated) control

were set at 100% viability, and readings from cells treated with NB19, a mixture of NB19 and Ang1, or Ang1 alone were expressed as % of the control. Specifically, percent of cell viability was determined from the equation $(A_t - A_b)/(A_c - A_b) \times 100$, where A_t is the absorbance of NB19, A_b is the absorbance of the blank (medium alone), and A_c is the absorbance of the untreated control [37].

Apoptosis analysis

TIME and U-87-MG cells were seeded at 5×10^4 cells per well in 24-well Nunc™ cell-culture treated plates (Thermo Scientific) and then incubated overnight under 5% CO₂ in complete growth medium, i.e., Vascular Cell Basal Medium (ATCC) or Minimum Essential Medium Eagle, respectively, at 37 °C. The growth medium was then replaced with 250 μ L of starvation medium (2% FBS) containing 30 nM or 300 nM of Nb19 or 500 μ M Na₂SeO₃ or no additions (control), all with or without 500 ng/mL Ang1. The cells were incubated overnight before trypsinization with 100 mL Trypsin B (Biological Industries, IL, USA), followed by the addition of 1 mL of starvation medium, transfer into Eppendorf tubes and centrifugation at 200g for 5 min. The supernatant was discarded, and the cells were washed with Annexin binding buffer before staining with an Annexin V APC and SYTOX Green dye mixture (Dead Cell Apoptosis Kit, Thermo-Fisher), according to the manufacturer's protocol. Additional wells were incubated with 2 mM H₂O₂ for 4 h (instead of overnight) and were then processed similarly. Apoptotic and permeabilized cells were counted by red and green fluorescent signals, respectively, by a BD FACSCanto™ II Flow Cytometry System (BD Biosciences, San Jose, CA, USA), and the mean fluorescence levels were recorded.

Wound-healing assay

An in vitro wound-healing assay was performed as previously described with some modifications [38]. Briefly, U87-MG cells were seeded in 24-well plates at a density of 1.5×10^5 cells in each well and allowed to grow for 24 h at 37 °C until they reached confluence. A linear scratch was created in each confluent monolayer by gently scraping the surface with a sterile p200 pipette tip (care was taken during scratching process to ensure similar scratches for all samples). The scratched monolayers were then washed twice to remove nonadherent cells. Ang1 (500 ng/mL)-treated cells were used as the positive control, and untreated cells, as the negative control. Test samples included mixtures of 30 nM (or 300 nM) of NB19 and Ang1 (500 ng/mL). Images were taken exactly at the same position prior to and post incubation of cells with the different treatments or controls. Wounds were viewed under a light microscope immediately

after the scratches were made and again after 6, 12, 18 and 24 h of incubation at 37 °C. The experiment was performed in triplicate, and the images were analyzed using ImageJ.

Statistical analysis

Data were analyzed with GraphPad Prism version 9.00 for Windows (La Jolla, CA, USA). Standard deviation (SD) was determined from at least three independent experiments. Comparisons between the groups were performed using a t test (for two independent samples) and with one-way ANOVA (for multiple groups). A probability value of <0.05 was considered statistically significant.

Results

Screening the Naïve NBlib library for nanobodies with a high affinity to Tie1–ECD

To produce highly expressed stable nanobodies exhibiting affinity to Tie1, three approaches were taken. In the first approach, a synthetic (non-immune) naïve nanobody library, designated Naïve NBlib, was generated by randomizing (using NNS degenerate codons) specific positions within the three CDR loops (i.e., CDR1 at positions 25–34, CDR2 at positions 52–68, and CDR3 at positions 98–114) of a single nanobody clone that was previously isolated by the BGU group from a camel-derived nanobody library [39]. This parental nanobody clone was shown to be highly stable, and its solved crystal structure enabled us to accurately identify CDR positions that were likely to tolerate mutagenesis with only a minimal effect on the structure and stability of the mutated nanobody [39]. The Naïve NBlib library that was generated from four assembly PCRs of NNS-bearing DNA ultramer pairs was transformed into yeast, yielding 10^8 variants (Supplementary Fig. S1). The Naïve NBlib library was then sorted for high expression and increased affinity to Tie1–ECD using seven consecutive rounds of affinity sorts, each one with a decreasing concentration of Tie1 (Supplementary Fig. S2). To identify high-affinity nanobody variants, 20 individual clones were randomly isolated from the seventh affinity sort. The level of binding to Tie1 of each of these 20 nanobody variants (normalized to their expression level) was then determined (Supplementary Fig. S3A). Sequencing analysis of the 10 variants that showed the highest affinity to Tie1 revealed unique sequences that were repeatedly identified in two variants (Supplementary Fig. S3B), namely, clone 7.16 with an apparent K_D of 12.81 nM and clone 7.20 with an apparent K_D of 15.72 nM; the K_D values were estimated from titrations of clones 7.16 and 7.20 displayed on the yeast surface for affinity to soluble Tie1 (Supplementary Fig. S3C). Nevertheless, our attempts to

produce these nanobody variants as soluble proteins were unsuccessful due to their lack of expression or low proteolytic stability in *Saccharomyces cerevisiae*.

Treating the Naïve NBlib library with trypsin or chymotrypsin before sorting produces proteolytically stable nanobody variants

To select nanobody clones likely to show high expression, proper folding, and stability (to both heat and proteolysis), our second approach comprised treating the parental Naïve NBlib library, prior to affinity screening, with a protease according to the protocol of Rocklin et al. [34]. This methodology was chosen as a means to eliminate unfolded protein variants, which are particularly susceptible to cleavage (Supplementary Fig. S4A) [34]. After treating Naïve NBlib libraries with trypsin (Supplementary Fig. S4B) or chymotrypsin (Supplementary Fig. S4C) at various concentrations, flow cytometry analysis of the treated library clones labeled with a mouse anti c-myc antibody, followed by an anti-mouse antibody conjugated to PE, was performed to detect expression levels (as an indication of the complete amino acid sequence and correct folding) (Supplementary Fig. S4B, C). The conditions giving the highest expression levels, namely, chymotrypsin as the protease and a concentration of 30 nM, were chosen for sorting the Naïve NBlib library to identify stable nanobodies. This treatment gave an expression level of the library of 10%, as shown in the red fluorescence signal in Supplementary Fig. S5A (indicated by the black rectangle). The expressing population was sorted to yield a library, designated CT-NBlib, having a size of 10^6 (represented by the blue fluorescence signal in Supplementary Fig. S5A). The CT-NBlib library was then subjected to affinity maturation for binding to Tie1 using decreasing concentrations of Tie1 for each sorting gate (Supplementary Fig. S5B–F). From the last sort, 20 individual clones conferring the strongest affinity to Tie1 were isolated (Supplementary Fig. S5G). The apparent affinity of each YSD nanobody clone to Tie1 was determined, and the most potent clones were sequenced and cloned into an expression vector. Here again, our attempts to produce these variants as soluble proteins were unsuccessful due to their lack of expression or low stability in *S. cerevisiae*.

Screening a predesigned nanobody library against Tie1 produced a nanobody exhibiting high expression and high affinity for Tie1

In our search for a stable nanobody exhibiting high affinity to Tie1 and high expression, our third approach was based on the naïve, but predesigned, synthetic library that was previously developed by McMahon et al. using a combination of computational and experimental methods [34]. In this

nanobody library, designated Predes NLib, positions in the CDR loops had been optimized for expression and stability of the nanobodies. Predes NLib was expressed in *S. cerevisiae* using the pYDS649 plasmid [34], with expression of the library (on the cell wall) being detected by targeting the HA-tag expressed at the C-terminus of the nanobody library (Fig. 1a). The Predes NLib library, with a size of 10^8 , was then sorted for Tie1 binding using the same YSD method as that applied for the Naïve NLib library (Fig. 1B). Following the final (i.e., fifth) round of affinity sorting against Tie1 as the target, 20 individual nanobody clones were randomly selected and isolated, and the affinity of each clone for soluble Tie1 (25 nM) was determined (Fig. 1c). Nine clones with high binding (normalized to expression) levels were sequenced. Among these clones, the optimal pI value for production—in terms of optimal secretion and low interaction with the yeast membrane during production [40]—i.e., pI 4.96 (Supplementary Table S2), was exhibited by clone NB19. This clone was successfully produced recombinantly in *Pichia pastoris* strain GS115 and purified by affinity chromatography, followed by SEC (Supplementary Fig. S6A, B). The yield was 1 mg/mL of cultured yeast. SDS-PAGE and mass spectrometry (MS) analysis confirmed that NB19 was pure (Supplementary Fig. S6A, B), and had been produced

with the correct molecular weight of 15.7 kDa (Supplementary Fig. S6C).

The purified NB19 nanobody binds Tie1 with high affinity

The selectivity of the binding of NB19 for Tie1 (vs. Tie2) is reflected by the K_D values—calculated from the SPR results—for the binding of immobilized NB19 to Tie1-ECD and Tie2-ECD, namely, 0.170 nM (K_{on} 1.09×10^5 M⁻¹ s⁻¹; K_{off} 1.86×10^{-5} M⁻¹ s⁻¹) and 6 nM (K_{on} 6.85×10^4 M⁻¹ s⁻¹; K_{off} 4.18×10^{-4} M⁻¹ s⁻¹), respectively (Fig. 2a, b). The same binding tendency was found for the binding of NB19, at different concentrations, to HEK293 cells transfected with Tie1, Tie2, or both (Fig. 2C), with K_D values for HEK293 cells overexpressing Tie1 or Tie2 being 0.09 nM and 10 nM, respectively (Fig. 2D and Supplementary Fig. S7). Of note, an excellent fit ($R^2 = 0.978$) was obtained for a two-phase binding model in HEK293 cells expressing both Tie1 and Tie2, reflecting the differences in affinities for the two receptors. The apparent K_D values of NB19 binding to U87-MG and TIME cells were 1.5 nM and 1.8 nM, respectively. Microscopy experiments confirmed the binding of NB19 to Tie1 and Tie2 (Fig. 2E) with colocalization Manders coefficients of 0.78 and 0.79 for Tie1 and Tie2, respectively

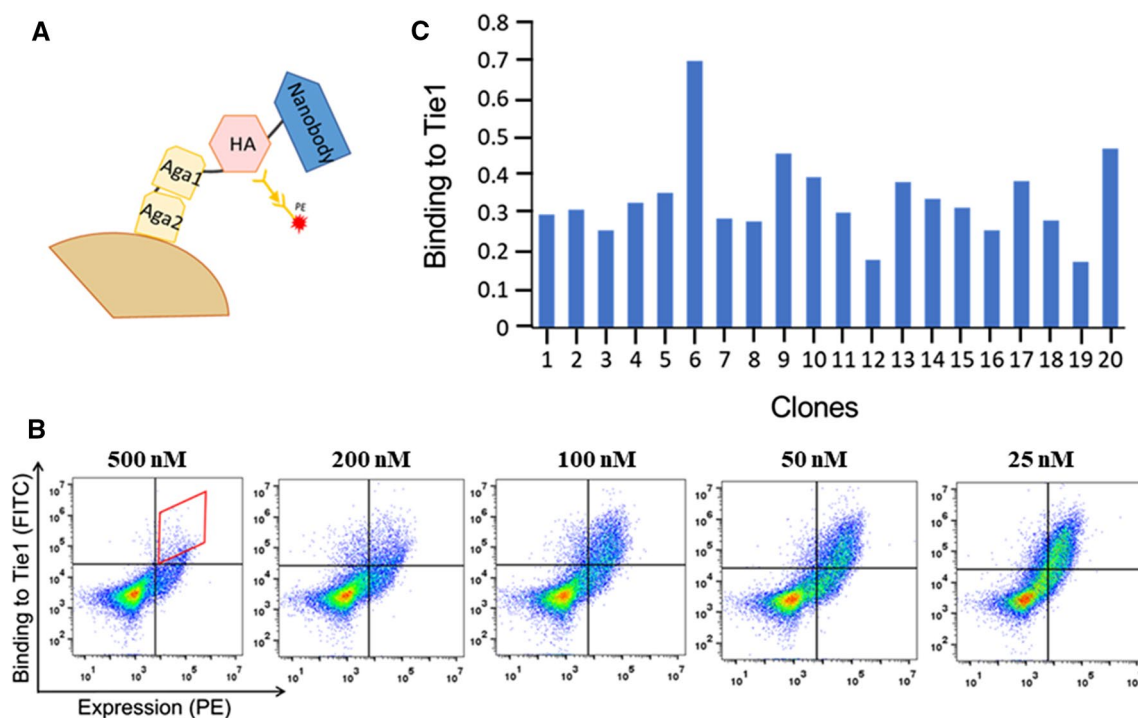


Fig. 1 Screening of the computationally designed Predes NLib library for binding to Tie1. **a** Schematic representation of the Predes NLib library in the YSD format. **b** Analysis of nanobody library expression and binding to 100 nM Tie1 after sorting against Tie1 at concentrations of 500, 200, 100, 50, and 25 nM (indicated above each

panel). The sorting gate for the first sort is shown in red, and gates of the other sorts were identical to the gate shown in (a). **c** Binding to Tie1 normalized to expression levels of 20 randomly selected nanobody clones from the fifth sort. The Tie1-binding fluorescence signal for each nanobody clone was divided by its expression signal

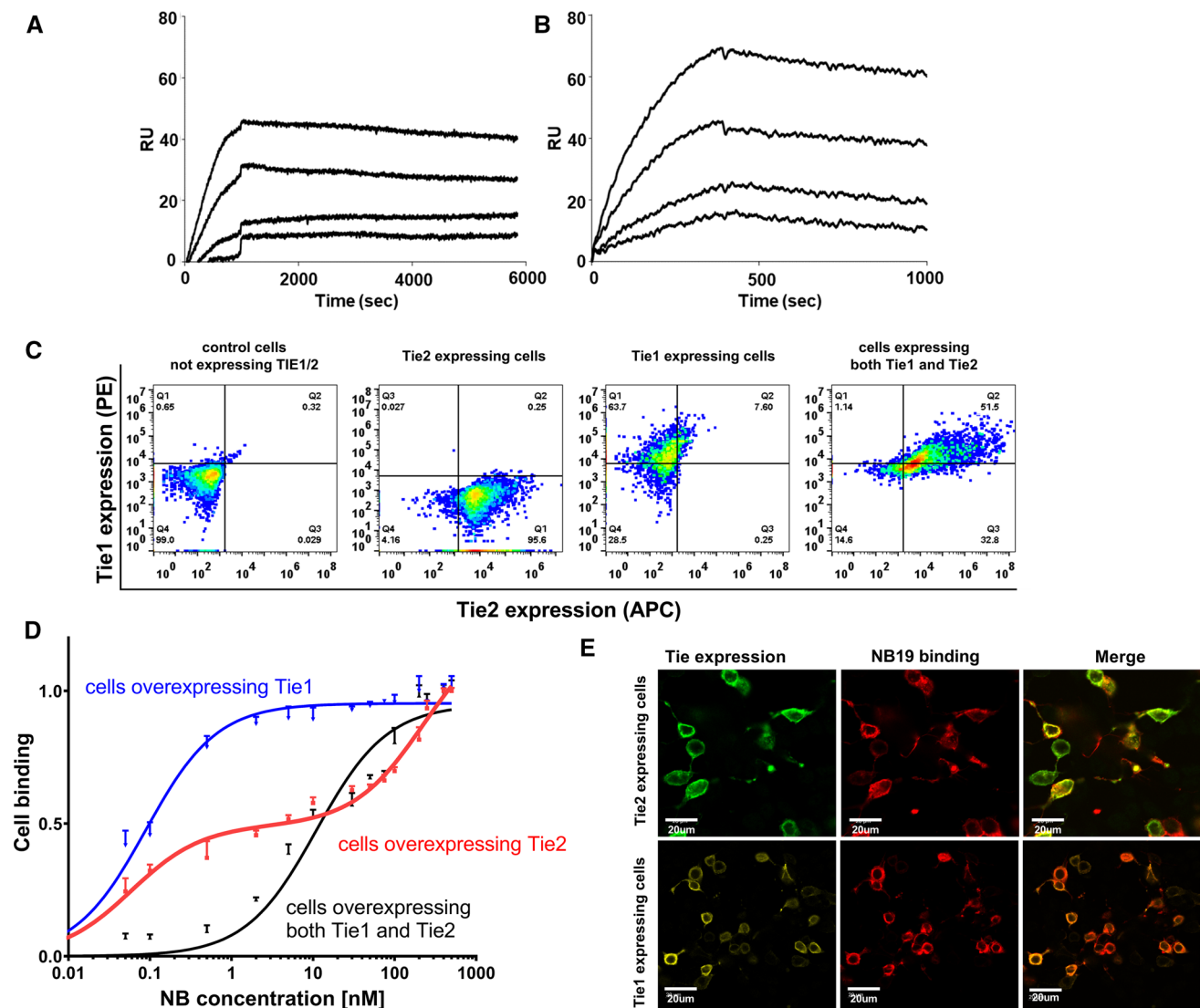


Fig. 2 Binding of NB19 to recombinant and overexpressed Tie1 and Tie2. **a** Representative SPR sensorgrams for binding of Tie1–ECD (a) and Tie2–ECD (b) to immobilized NB19. Different concentrations of Tie1–ECD and Tie2–ECD were allowed to flow over immobilized NB19 for 400 s, and dissociation was monitored for 60 min. **c** Cell surface expression analysis of Tie2 and Tie1 in transfected vs. untransfected HEK293 cells. HEK293 cells were stained with APC-labeled anti-human Tie2 and PE-labeled anti-human Tie1 antibodies

and incubated at 4 °C for 30 min before analysis by flow cytometry. **d** Affinity titration curves of NB19 binding to HEK293 cells overexpressing Tie1, Tie2, or both Tie1 and Tie2. **e** Confocal fluorescence microscopy images showing colocalization of NB19 and Tie1 and Tie2 receptors in HEK293 cells overexpressing these receptors. Cell surface expression of Tie2 (green-488) and Tie1 (yellow-PE) are shown; the binding of NB19 was detected by APC anti-his antibody

(Supplementary Fig. S8). As expected, no binding was observed for NB19 to untransfected HEK293 cells. Similarly, affinity titration curves of NB19 binding to glioblastoma U87-MG or human endothelial TIME cells, which endogenously express both Tie1 and Tie2, also showed an excellent fit ($R^2=0.98$ and $R^2=0.97$ for U87-MG and TIME cells, respectively) to a two-phase binding model (Fig. 3).

Tie1-dependent inhibition of the phosphorylation of RTKs in TIME cells

The next set of experiments was designed to test the phosphorylation dependency of Tie2 (and other RTKs) on Tie1, and the effect of NB19 on RTK activation. For this purpose, three specific siRNAs were used (i.e., 13.1, 13.2 and 13.3) to knock down Tie1 expression in TIME cells (Supplementary Fig. S9). As expected, treatment with siRNA reduced Tie1 expression levels in non-supplemented conditioned medium (Supplementary Fig. S9) and in conditioned

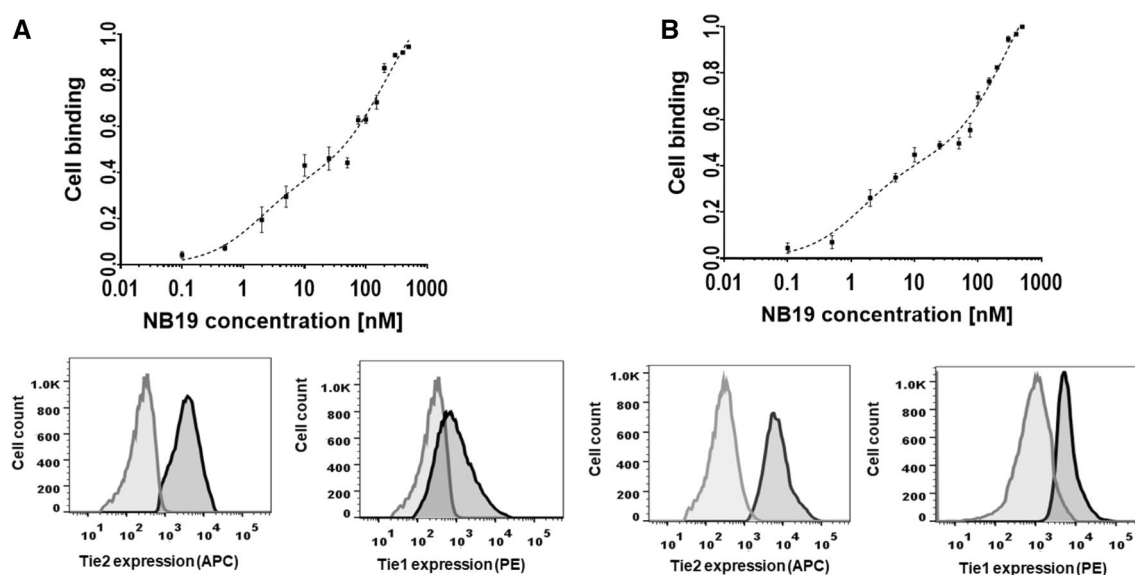


Fig. 3 NB19 binding to cells endogenously expressing Tie1 and Tie2. *Top panel:* Affinity titration curves of NB19 binding to U87-MG cells (a) and TIME cells (b). Two-phase binding curves are shown with $R^2=0.98$ and 0.97 , respectively. *Bottom panel:* Cell surface expression of Tie2 and Tie1 in stained (dark gray) U87-MG cells (a) and

TIME (b) cells is presented in comparison with unstained cells (light gray). In both cases, cells were stained with APC-labeled anti-human Tie2 antibody and PE-labeled anti-human Tie1 antibody and incubated at 4°C for 30 min before analysis by flow cytometry

medium supplemented with Ang1 (Fig. 4a) compared with the expression levels of the negative control (NC)-siRNA-untreated cells. In support of Tie1/Tie2 cross-talk [4], a significant reduction was also obtained for Tie2 phosphorylation upon silencing Tie1 expression in both untreated and Ang1-treated cells (Fig. 4B). Furthermore, Ang1-dependent phosphorylation of Akt and Fak, which are downstream of Tie1 in this activation axis, was significantly decreased following the silencing of Tie1 expression (Fig. 4c, d). Of note, similar to the effect of silencing Tie1 expression (by siRNA) on the phosphorylation of RTKs (i.e., Tie2, Akt and Fak), a dose-dependent inhibition of the phosphorylation of these RTKs and of Tie1 was observed upon treatment with NB19 (Fig. 5).

NB19 inhibits tube formation in endothelial cells

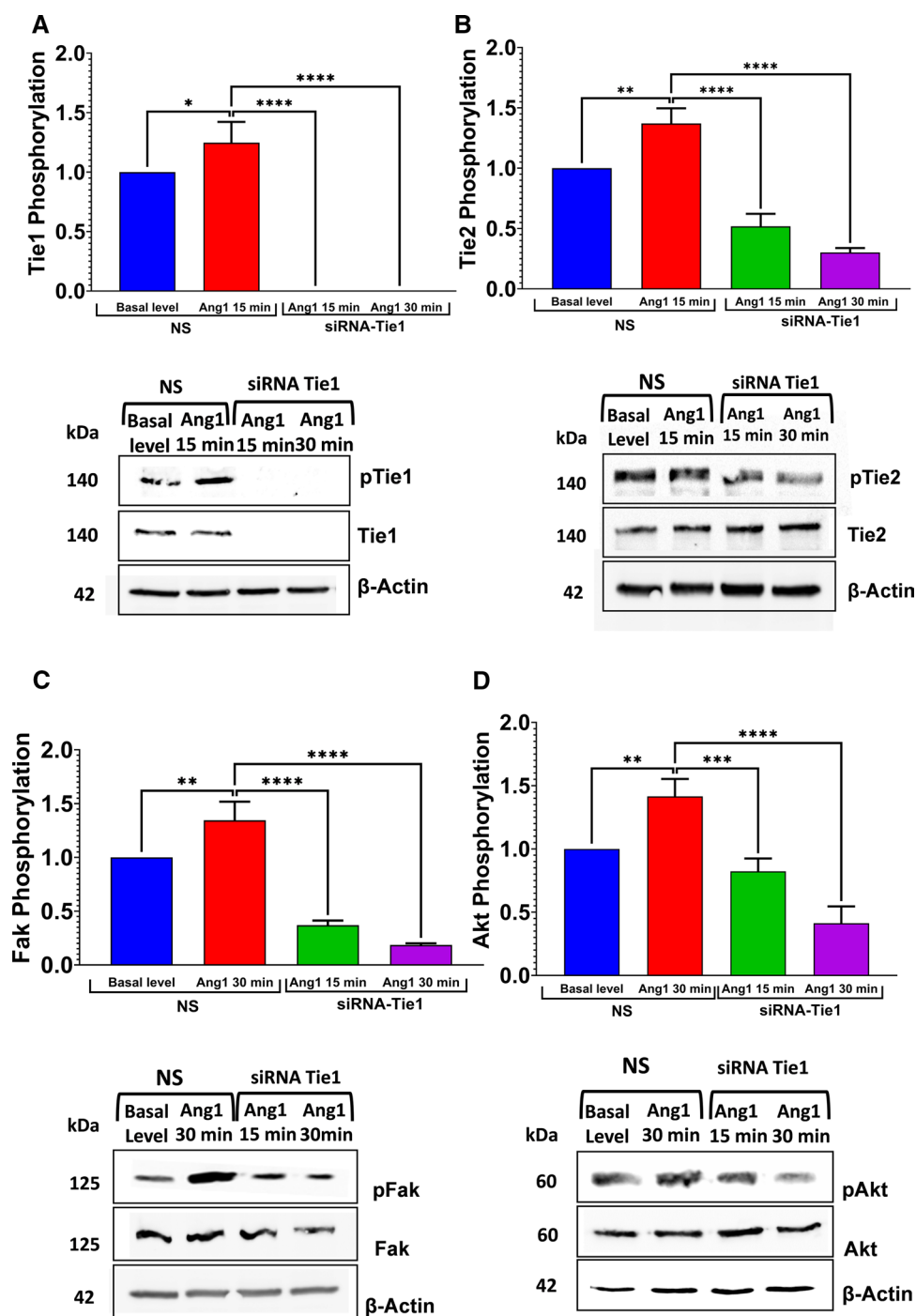
To test whether NB19 inhibits Tie1-dependent tube formation, TIME cells were seeded on a layer of pre-solidified Matrigel in a 24-well plate. Following 18 h of incubation with NB19, several parameters of the tube network were quantified using confocal microscopy imaging and computed software analysis (Fig. 6a–c). Treating cells with 30 nM of NB19 (the concentration at which it primarily binds to Tie1) or with 300 nM (the concentration at which it binds both Tie1 and Tie2) led to a decrease in the total tube length and in the number of junctions, in a dose-dependent manner, with levels falling below the basal value (Fig. 6b, c). Even at the basal level (without adding external Ang1), endogenous

Ang1 caused the formation of tubes, and therefore, when the nanobody was added, there was a decrease to below the basal level. Cell aggregation into tube-like structures started immediately after seeding of cells, and defined tube-like structures became visible after 4 h. A real-time experiment showed that inhibition of tube formation by NB19 started as early as 2 h post-treatment, namely, at the same time that the tube started to form in the untreated control samples (1–2 h after plating); this inhibition of tube formation in the NB19-treated (but not in the untreated control) cells continued throughout the entire 24-h experiment (movie in Supplementary Material). In addition to its effects on tube formation, NB19 caused a decrease in endothelial cell viability—by 20% at a low NB19 concentration (30 nM) and by 50% at a high concentration (300 nM), as determined by the XTT assay (Fig. 6d).

NB19 suppresses cancer cell migration

U87-MG glioblastoma cells were used to test whether NB19 treatment could inhibit the Ang1-stimulated migration of cancer cells. Cell migration was found to be significantly inhibited, with the wound area exhibiting a ~65% cell-free area when treated with 30 nM NB19 compared to control (~7% cell-free area). This inhibitory effect was dose-dependent, such that ~80% of the wound area remained cell free upon treatment with 300 nM NB19 (Fig. 7a, b). As shown in Fig. 7a, b, migration of Ang1-stimulated U87-MG cells was reduced by 45% following a 24-h treatment with 30 nM

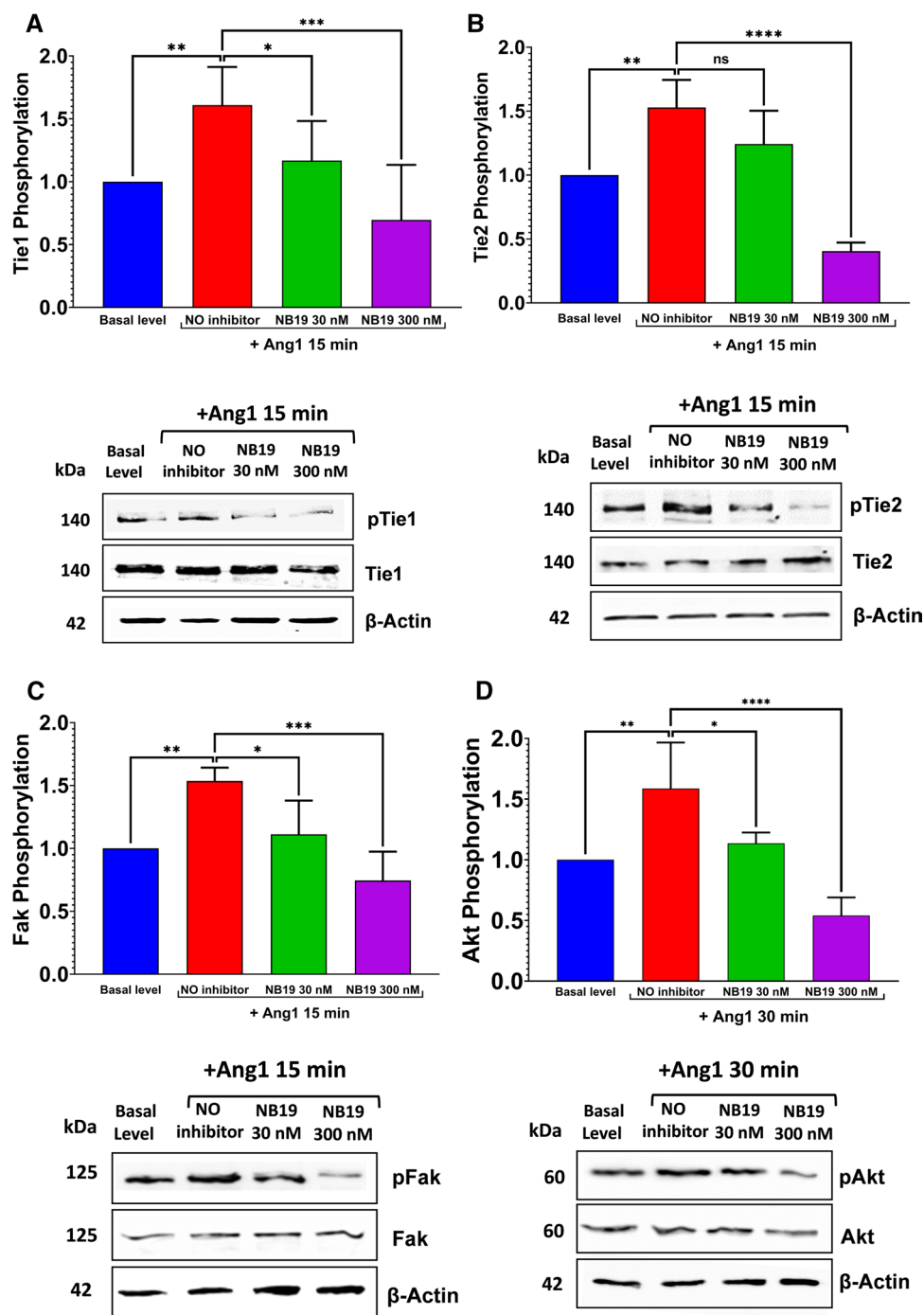
Fig. 4 Tie1-dependent inhibition of phosphorylation of RTKs in TIME cells. **a** Determination of Tie1 expression and phosphorylation under different conditions: TIME cells were transfected with siRNA-Tie1 or scrambled siRNA (designated NS) and then treated with control buffer (basal level) or 500 ng/mL Ang1 for 15 or 30 min in a 12-well Multidish plate. Cell lysates were analyzed by western blot using antibodies against phosphorylated Tie1 (pTie1), Tie1, and β -actin. **b–d** Phosphorylation of Tie2, Fak, and Akt, respectively, was determined as in panel (a). In each case, cell lysates were analyzed by western blot using antibodies against the relevant phosphorylated kinase (pTie2, pFak, or pAkt), the kinase itself (Tie2, Fak, or Akt), and β -actin. The intensity of each phosphorylated band measured by ImageJ software was normalized to the expression of total protein of each experiment, and this value was subsequently normalized to the total quantity of β -actin for each sample. Error bars represent SD. One-way ANOVA with Dunnett's multiple comparison to the Ang1-only treatment was utilized for statistical analysis; * $p < 0.1$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. NS, non-silenced



NB19. Furthermore, this difference in migration (40–50% reduction following treatment with NB19) remained constant for all time points tested. For treatment with 300 nM, the NB19 migration distance was reduced by 65% after a 24-h treatment. This finding showed that NB19 reduced the U87-MG cell migration distance in a concentration-dependent manner and that a low concentration of NB19 was sufficient to inhibit most of the migration. For understanding

whether the inhibitory effect of NB19 on cell migration was the result of the inhibition of cell migration per se or the result of a decrease in cell viability, we used a viability assay, namely, the XTT assay. The results showed a non-significant decrease in U87-MG cell viability upon treatment with a low concentration of NB19 (30 nM) and 30% inhibitory effect upon treatment with a high concentration of NB19 (300 nM). There was no decrease in cell viability

Fig. 5 Inhibition of Tie1 and Tie2 phosphorylation by NB19 in TIME cells. **a** Determination of Tie1 phosphorylation: TIME cells were treated with buffer (control, basal level), 500 ng/mL Ang1, a combination of 500 ng/mL Ang1 and 30 nM/300 nM NB19 for 15 min in a 12-well Multidish plate. Cell lysates were analyzed by western blot using antibodies against phosphorylated Tie1 (pTie1), Tie1, or β -actin. **b–d** Phosphorylation of Tie2, Fak, and Akt, respectively, was determined as in A, with the exception that the incubation time for Akt was 30 min. Cell lysates were analyzed by western blot using antibodies against the relevant phosphorylated kinase (pTie2, pFAK, or pAkt), the kinase itself (Tie2, Fak, or Akt), or β -actin. The intensities of the phosphorylated bands measured by ImageJ were normalized to the expression levels of total protein in each sample, and this value was subsequently normalized to the total quantity of β -actin for each sample. Error bars represent SD. One-way ANOVA with Dunnett's multiple comparison to the Ang1 treatment was utilized for statistical analysis; * $p < 0.1$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. ns, non-significant



below the basal level, unlike the decrease in cell migration, which means that at both concentrations of NB19 (30 and 300 nM), there was an inhibition of cell migration that was not dependent on cell viability.

The results also show that upon treatment with NB19, there was a minor (non-significant) Ang1-dependent increase in apoptosis and cell permeability in endothelial cells. In

U87-MG cells upon treatment with NB19, there was no effect on apoptosis, but there was an Ang1-dependent increase in cell permeability (Supplementary Fig. S10). Of note, these effects of NB19 may result from its ability to interact with Tie2 (that has Ang1-dependent anti-apoptotic and anti-permeability effects [41]).

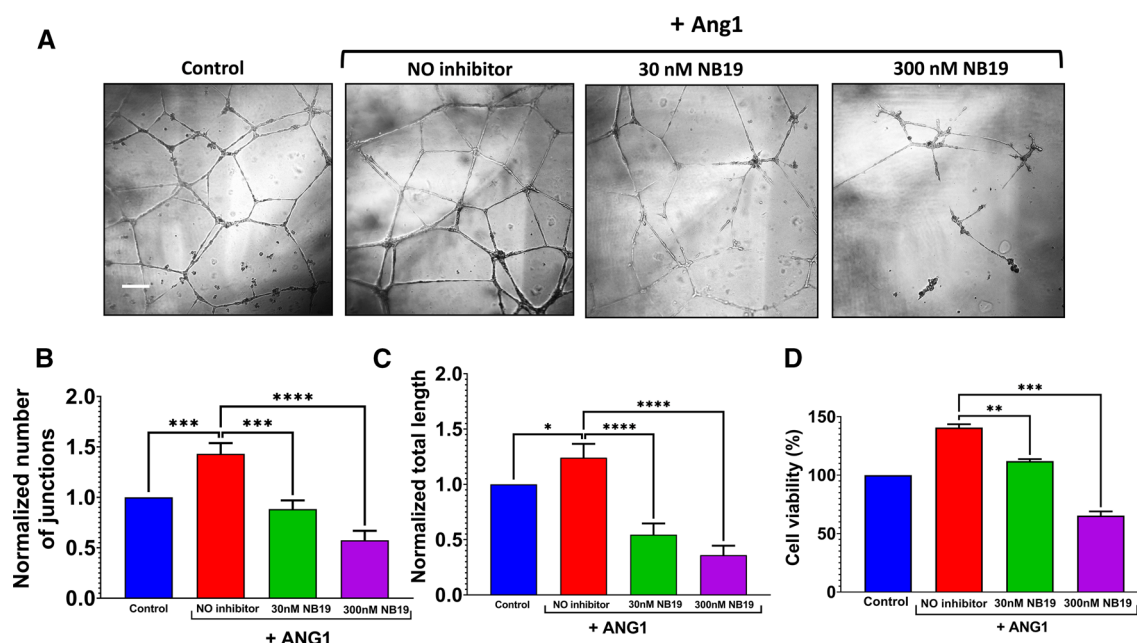


Fig. 6 Inhibition of tube formation in endothelial cells by NB19. **a** TIME cells were seeded at 1×10^5 in 8-well plates coated with Matrigel and imaged using confocal microscopy following 18 h of treatment under the conditions indicated in the top panels. Scale bar, 100 μ m. **b, c** Tube structures were analyzed for the number of generated junctions and the total tube length following the different treat-

ments. * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$, $n = 3$. **d** Effects of NB19 on the growth and survival of TIME cells, as reflected in the number of viable cells assessed by an XTT assay. The results for the different samples were normalized to the untreated control. * $p < 0.1$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, $n = 3$

Discussion

Any study of an orphan receptor, such as Tie1, as a therapeutic target poses experimental challenges, since the lack of cognate ligands complicates the elucidation of its pharmacology, biological functions, and therapeutic potential. This challenge may be overcome by generating high-affinity camelid-derived nanobodies of a size and topography that make them accessible to the orphan target. Since no Tie1 functional epitopes are known, we sought to develop a nanobody that would act as a binder for Tie1, with the choice of a nanobody—rather than a conventional antibody—being dictated not only by the better accessibility of nanobodies but also by the common restriction preventing antibodies, but not nanobodies, from binding conserved epitopes due to immunological tolerance of self-antigens. As conserved epitopes often drive key protein functions, such as the interaction of Tie1 with Tie2 or other effector proteins not yet identified, and allosteric communication with such proteins, a rapid method using a yeast-based selection scheme (enabled by FACS) to identify nanobodies that target such sites provides a robust means to interrogate Tie1 function and was thus used in this study.

The screening protocol developed in this study derives from previous studies using nanobody display technologies (e.g., for preparing whole-cell biosensors) that are

based on bacterial or yeast cells [42]. Once displayed on the surface of bacterial or yeast cells, nanobodies fold into their native and functional structure. Yeast surface display has thus been used for screening nanobody libraries, which is followed by affinity sorting of selected clones by flow cytometry [34, 43–46]. Nevertheless, to date, the above strategies have been tested on immune, animal-derived nanobody libraries, which restricts their use with synthetic libraries. Another inherent problem of previously developed strategies is that, in many cases, there is not a good correlation between the expression and function of the displayed nanobody variant and its expression and function as a purified protein. This latter disadvantage is especially true for universal, non-animal-derived nanobody libraries, i.e., the synthetic naïve libraries that are used when camelids are not available or, as in the case of Tie1, when the protein (i.e., the antigen) may be toxic to the camelid or is difficult to produce (limiting the quantities available for experimentation). In particular, nanobody variants that are selected from non-immune, synthetic library screens suffer from low expression yields and poor stability. To address these limitations, we developed a nanobody library selection platform designed to pinpoint a nanobody variant that would be able to inhibit, with strong potency and specificity, the Tie1 orphan receptor, while exhibiting good stability.

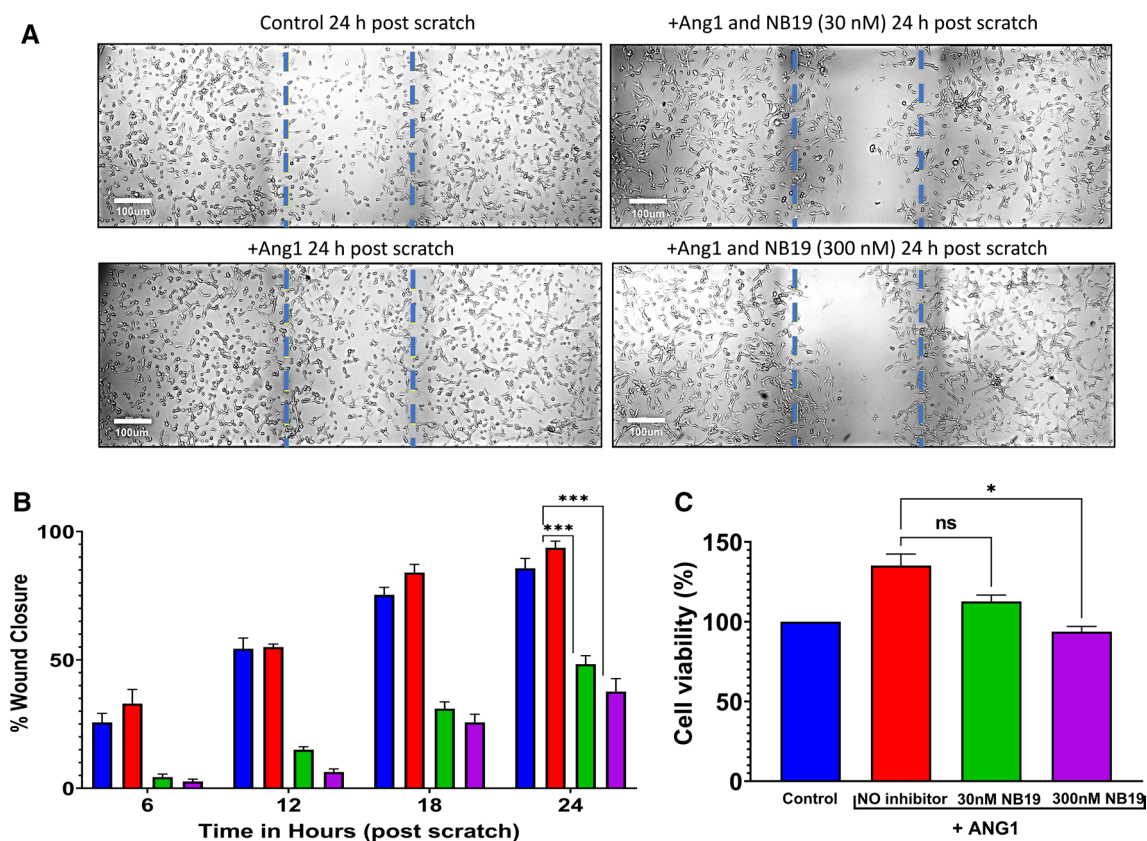


Fig. 7 NB19 suppresses cancer cell migration. A scratch assay was performed on U87-MG cells in the presence of 0, 30, or 300 nM NB19. **a** Representative photomicrographs of untreated and NB19-treated cells at 24 h post scratch. Broken blue lines indicate the migration front of cells. **b** Percentage of scratch closure at indicated time points during the assay. The bars are color coded as follows: blue—untreated control cells, red—Ang1-treated cells, green—

cells treated with 30 nM NB19 and Ang1, and purple—cells treated with 300 nM NB19 and Ang1. Error bars represent means $\pm$ SD of three scratched areas imaged per condition. *** $p < 0.001$. **c** Effects of NB19 on the growth and survival of U87-MG cells assessed by an XTT assay. The results for all samples were normalized to the untreated control. * $p < 0.05$, ns, non-significant. $n = 3$

Our working hypothesis at the start of the study was that stable high-affinity nanobody binders for Tie1 could be produced with high expression by screening Naïve NBlib, a library of clones randomized on their three CDR loops and based on a backbone sequence originating from a clone selected from a camel-derived immune library that had previously undergone stability screens [39]. However, although 20 individual clones were randomly selected and isolated from the seventh affinity sort against Tie1-ECD, attempts to produce the selected high-affinity Tie1-ECD variants as soluble proteins were unsuccessful due to a lack of expression in yeast—yet another indication of the challenging nature of the task of constructing stable nanobodies.

To overcome the problem of the lack of expression in a yeast-display system, the parental, naïve NBlib library was treated with proteases as means to select nanobodies that are properly folded and are hence stable toward degradation by proteases. This notion was based on a previous study, demonstrating that unfolded protein variants are susceptible

to cleavage by proteases, such as trypsin and chymotrypsin, since their exposed hydrophobic surfaces are readily available for digestion [32]. The resulting CT-NBlib library (pre-treated with chymotrypsin) was affinity matured for binding to Tie1-ECD to produce individual clones conferring strong affinity to Tie1-ECD. However, here again, as for the Naïve NBlib library, the individual clones were unstable as soluble proteins.

Being unable to produce nanobody variants that were stable and well expressed from the above naïve, randomly mutated libraries, we posited that the way forward would be to use a tailored (predesigned) nanobody library with CDR amino acid compositions that had previously been optimized for high stability and expression. In fact, there are a number of reports of the retrieval, from such predesigned nanobody libraries, of potent nanobodies that have been used in a variety of applications [36, 42–44]. To address the expression and stability challenges associated with our non-immune naïve library, we used the predesigned library (designated

Predes NBlib) developed by McMahon et al. [34]. Sorting of this library for high-affinity Tie1–ECD binding enabled us to isolate a stable, high expressing nanobody variant (i.e., NB19) that bound Tie1–ECD with a K_D in the subnanomolar range and that showed selectivity for Tie1–ECD over Tie2–ECD.

Having in hand, for the first time, a reagent (i.e., NB19) that binds to both Tie1 and Tie2 but with preferential binding to Tie1 over Tie2 and that strongly inhibits Tie1 enabled us to dissect out the roles of Tie1 and Tie2 in the Tie1–Tie2 signaling axis, especially the cross-talk between Tie1 and Tie2 and its influence on angiogenesis and cancer cell migration and viability. Having done so, we can perhaps reconcile studies producing conflicting results and also throw light on the commonly held belief that Tie1 modulation of Tie2 activity [4, 47] is context dependent. For example, one study showed that Ang1 induced the activation of both Tie1 and Tie2 by promoting the formation of a heteromeric Tie1 and Tie2 complex [16]. Other studies have indicated that Tie1 is an inhibitor of Tie2 [48, 49], and yet others have reported that Tie1 association with Tie2 is required for Tie2 activation by Ang1 in mice [16, 50] and that phosphorylation of Tie2 is reduced in Tie1-deleted versus control mice [5]. In the current study, we also show that inhibition of Tie1 by NB19 reduces Tie2 phosphorylation. We note that a similar inhibitory effect on Tie2 phosphorylation was obtained by inhibiting Tie1 through siRNA knock down.

Our results using NB19 are consistent with previous results and provide additional evidence that Tie1 is capable of activating both Fak and Akt to promote endothelial survival [1, 51]. Previous studies have indeed shown that, when activated, Tie1 may then activate Tie2 and other downstream effectors (i.e., Fak/PI3K/Akt pathway), but we show here for the first time that specific inhibition of Tie1 by NB19 results in a reduction of both, Fak/PI3K/Akt pathway activation and endothelial cell survival. Our results in cells also complement the previous reports that Tie1 influences the Tie2 signaling pathway and provide supporting evidence for the cross-talk between Ang1, Tie1 and Tie2, i.e., by showing that Tie1 is essential for the Tie2 agonistic activity of Ang1 [4].

It was also demonstrated in this study that NB19 significantly inhibits Tie1 and Tie2 phosphorylation in a dose-dependent manner and thus acts as a functional antagonist toward Tie1, subsequently leading to diminished cancer migration, growth, and angiogenesis. The ability of NB19 to bind both Tie1 and Tie2, but with preference for Tie1, was thus exploited to analyze the individual roles played by the two potential targets in pathological processes, including cancer angiogenesis, migration, and growth. NB19, at a low concentration (when bound predominantly to Tie1), was shown to inhibit the formation of the tubular structures in TIME endothelial cells, and a stronger inhibitory effect

was obtained at elevated NB19 concentrations. At these high concentrations of NB19, when Tie1 binding was at saturation levels, allowing NB19 to bind Tie2 as well, a stronger inhibition on the downstream processes (Akt and Fak phosphorylation, angiogenesis and cancer migration, and growth) was obtained. Further investigation of the complex and dynamic interactions between the Tie receptors and their molecular regulators and specific inhibitors (such as the nanobody developed in this study) will be critical for maximizing the therapeutic potential of these targets and their signaling pathways.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00018-022-04336-9>.

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Authors' contributions M.M., N.E., and N.P. designed the research; M.M., N.E., Z.A., and U.H. performed the research; M.M., N.E., Z.A., U.H., and N.P. analyzed the data; M.M. and N.P. wrote the paper. All authors edited the manuscript and approved the final version.

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Data availability All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Declarations

Competing interests The authors declare that they have no competing interests.

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