Pharmacodynamic and Antitumor Activity of BI 836880, a Dual VEGF and Angiopoietin 2 Inhibitor, Alone and Combined with Programmed Cell Death Protein-1 Inhibition

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Section: Drug Discovery and Translational Medicine

No. of text pages: 19
No. of tables: 1
No. of figures: 6
No. of references: 32

Abstract: 234/250 words
Introduction: 494/750 words
Discussion: 1,490/1,500 words

Abbreviations: DMEM, Dulbecco’s modified Eagle medium; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum FLD, fibrinogen-like domain; HEK293, human embryonic kidney cells; HRP, horseradish peroxidase; HUVECs, human umbilical vein endothelial cells; IC50, Half-maximal inhibitory concentration; IHC, immunohistochemistry; LL/2, Lewis Lung carcinoma; NSCLC, non-small cell lung cancer; PBS, Phosphate buffered saline; PFS, progression-free survival; PD-1, programmed cell death protein-1; PD-L1, programmed death-ligand 1; PlGF, placental growth factor; RMMM, repeated measurement mixed model; RTCA, real-time cell analysis; SmPC, summary of product characteristics; TGI, tumor growth inhibition; VEGF, vascular endothelial growth factor

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Keywords: angiogenesis, animal models, antibodies, anticancer agents, immunotherapy, programmed cell death, vascular endothelial growth factor
Abstract

VEGF and angiopoietin-2 (ANG2) have complementary roles in angiogenesis and promote an immunosuppressive tumor microenvironment. It is anticipated that combination of VEGF and ANG2 blockade could provide superior activity to blockade of either pathway alone, and that addition of VEGF/ANG2 inhibition to an anti-programmed cell death protein-1 (PD-1) antibody could change the tumor microenvironment to support T-cell-mediated tumor cytotoxicity. Here, we describe the pharmacologic and antitumor activity of BI 836880, a humanized bispecific nanobody™ comprising two single variable domains blocking VEGF and ANG2, and an additional module for half-life extension in vivo. BI 836880 demonstrated high affinity and selectivity for human VEGF-A and ANG2, resulting in inhibition of downstream signaling of VEGF/ANG2 and a decrease in endothelial cell proliferation and survival. In vivo, BI 836880 exhibited significant antitumor activity in all patient-derived xenograft models tested, showing significantly greater tumor growth inhibition (TGI) than bevacizumab (VEGF inhibition) and AMG386 (ANG1/2 inhibition) in a range of models. In a Lewis lung carcinoma syngeneic tumor model, combination of PD-1 inhibition with VEGF inhibition showed superior efficacy versus blockade of either pathway alone. TGI was further increased with addition of ANG2 inhibition to VEGF/PD-1 blockade. VEGF/ANG2 inhibition had a strong anti-angiogenic effect. Our data suggest that blockade of VEGF and ANG2 with BI 836880 may offer improved antitumor activity versus blockade of either pathway alone and that combining VEGF/ANG2 inhibition with PD-1 blockade can further enhance antitumor effects.

Significance statement (51/80 words)

VEGF and ANG2 play key roles in angiogenesis and have an immunosuppressive effect in the tumor microenvironment. Here, we show that BI 836880, a bispecific nanobody™ targeting VEGF and ANG2, demonstrates substantial antitumor activity in preclinical models. Combining VEGF/ANG2 inhibition with blockade of the PD-1 pathway can further improve antitumor activity.
Introduction

Blockade of VEGF signaling to restrict tumor vascular supply has long been recognized as a therapeutic strategy in oncology. The anti-VEGF monoclonal antibody, bevacizumab, in combination with chemotherapy, has been approved for the treatment of various advanced cancers, including renal cell cancer, non-small cell lung cancer (NSCLC), colorectal cancer and breast cancer (Avastin® [bevacizumab] SmPC 2021). Furthermore, numerous VEGF receptor tyrosine kinase inhibitors – including nintedanib, sorafenib, sunitinib, and pazopanib – are approved for the treatment of advanced cancers, including NSCLC, renal cell carcinoma, hepatocellular carcinoma, thyroid carcinoma, gastrointestinal stromal tumors, and pancreatic neuroendocrine tumors (Vargatef® [nintedanib] SmPC 2021; Nexavar® [sorafenib] SmPC 2021; Sutent® [sunitinib] SmPC 2021; Votrient® [pazopanib] SmPC 2021).

While tumors are often highly vascularized, the structure and function of vessels are often abnormal. It is thought that these abnormalities can contribute to tumor progression and metastasis, for example, by migration of tumor cells through leaky vessels (Carmeliet and Jain, 2011). Thus, there is increasing interest in vessel normalization as a complementary therapeutic strategy to VEGF signaling blockade. The angiopoietin (ANG)/TIE2 pathway regulates vessel normalization. ANG1/TIE2 signaling promotes the interaction between endothelial cells and pericytes, stabilizing the maturing vascular system. ANG2 – the antagonist of ANG1 – destabilizes established blood vessels and promotes vessel remodeling and angiogenesis (Carmeliet and Jain, 2011; Gerald et al., 2013). There is also crosstalk between the VEGF and ANG2 signaling pathways: VEGF upregulates ANG2 expression in endothelial cells (Reginato et al., 2011) while ANG2 sensitizes endothelial cells to VEGF signaling (Fiedler and Augustin, 2006). Accordingly, combined inhibition of VEGF and ANG2 has demonstrated improved antitumor efficacy versus VEGF or ANG2 inhibition alone in preclinical models (Brown et al., 2010; Hashizume et al., 2010).
In addition to driving tumor neovascularization, pro-angiogenic factors, such as VEGF, promote an immunosuppressive environment within tumors, by inhibiting dendritic and cytotoxic T cell function and by activating regulatory T cells/myeloid derived suppressor cells (Yang et al., 2018). VEGF-A has also been described to interfere with T cell infiltration into the tumor (Motz et al., 2014). Moreover, abnormal vasculature of tumors is thought to suppress antitumor immunity by creating a hypoxic environment that interferes with T-cell function (Lanitis et al., 2015). Therefore, it may be beneficial to combine VEGF/ANG2 blockade with an anti-programmed cell death protein-1 (PD-1)/PD-L1 antibody. It is anticipated that such a combination could change the tumor microenvironment to support T-cell-mediated tumor cytotoxicity.

BI 836880 is a humanized bispecific nanobody comprising two single variable domains binding VEGF and ANG2 and an additional serum albumin-binding module for extending the half-life in vivo. Based on i) Biacore surface plasmon resonance binding assays; ii) cell proliferation/ERK phosphorylation assays in human umbilical vein endothelial cells (HUVECs) and iii) human TIE2 phosphorylation assays in transfected HEK293 cells, we assessed the specificity and pharmacologic activity of BI 836880 in vitro. We also evaluated its antitumor activity in a series of patient-derived xenograft models and the combination of VEGF/ANG2 inhibition with PD-1 inhibition in a Lewis Lung carcinoma (LL/2) syngeneic mouse tumor model.

**Materials and Methods**

**Reagents and cell lines**

BI 836880 and AMG386 (anti-ANG1/2 peptibody) were produced in-house by Boehringer Ingelheim. Other reagents were: RMP 1-14 (murine anti-PD-1 antibody; BioXCell; BE0146) and the corresponding isotype InVivoMAb Rat IgG2a (BioXCell; BE0089), bevacizumab (anti-VEGF antibody; Roche), and vatalanib (small-molecule tyrosine kinase inhibitor of VEGF receptor [VEGFR]; MedChemExpress; HY-12018).
For the binding assays, the following commercial recombinant proteins were used: human VEGF165 (R&D Systems; 293-VE/CF), human VEGF121 (R&D Systems; 298-VS/CF), rat VEGF164 (R&D Systems; 564-RV/CF), mouse VEGF164 (R&D Systems; 493-MV/CF), human VEGF-B (R&D Systems; 751-VE), human VEGF-C (R&D Systems; 2179-VC/CF), human VEGF-D (R&D Systems; 622-VD/CF), human P1GF (R&D Systems; 264-PG/CF), FLAG-tagged human ANG1 (Alexis Biochemicals), FLAG-tagged human ANG2 (Alexis Biochemicals), human TIE2-Fc (R&D Systems; 313-TI) and recombinant human ANG2 (R&D Systems; 623-AN). Also utilized were recombinant proteins produced by Boehringer-Ingelheim: mouse, rat, and cynomolgus ANG2-fibrinogen-like domain (FLD) fragments, and monomeric ANG2-FLD, which consists of the C-terminal FLD domain of ANG2 and contains the key amino acid residues involved in its interaction with the receptor TIE2. An anti-FLAG-horseradish peroxidase (HRP; Sigma), an anti-V_{HH} antibody (1A4; Ablynx) and a non-binding V_{HH} antibody control (Ablynx) were also used.

The antibodies used in the phosphorylation assays were: phospho-ERK-specific primary antibody (Sigma; M8159), anti-TIE2 antibody (Cell Signaling; 4224), secondary anti-phosphotyrosine HRP-conjugate 4G10 platinum (Millipore; 16-316).

HUVECs (Lonza Bioscience; 171747) were cultured in Endothelial Cell Growth Medium-2 (Endothelial Cell Growth Basal Medium-2 + 2% FBS + growth factors; Lonza Bioscience BulletKit) for up to eight passages. The human embryonic kidney cell line HEK293 was cultured for up to 20 passages in DMEM (Dulbecco’s modified Eagle medium; Lonza Bioscience) supplemented with 1 mM sodium pyruvate (Gibco), 1×MEM NEAA (Gibco), 12.5 mM HEPES pH 7.3 (USB), 100 U/mL Penicillin/Streptomycin (Gibco), and 10% heat-inactivated FBS (JRH Biosciences). Cell culture was performed at 37°C in a humidified incubator containing 5% CO₂. Cell lines were authenticated by Short Tandem Repeat analysis and regularly tested for Mycoplasma contamination.

**Binding affinity (Biacore analysis)**
Binding kinetics of BI 836880 to ANG2 and serum albumin were analyzed using a Biacore T100 surface plasmon resonance system. Briefly, ANG2 or serum albumin were immobilized directly on a CM5 chip via amine coupling. Nanobodies were analyzed at concentrations of 0.4–100 nM for ANG2, and 2–500 nM for serum albumin. Association/dissociation data were evaluated by fitting a 1:1 interaction model (Langmuir binding) or Heterogeneous Ligand model for ANG2 and serum albumin.

**Binding affinity (enzyme-linked immunosorbent assay [ELISA])**

The binding affinity of BI 836880 to recombinant human VEGF165, VEGF121, mouse VEGF164, rat VEGF164, human VEGF-B, human VEGF-C, human VEGF-D, and human placental growth factor (PIGF) was determined by ELISA. One µg/mL or 100 ng/well of each protein was directly immobilized in a 96-well MaxiSorp™ plate (Nunc; 430341) and blocked with 250 µL blocking buffer. Serial dilutions of test articles were prepared in assay buffer. Bound test articles were detected using biotinylated anti-VHH 1A4 antibody followed by ExtrAvidin-HRP for detection. Positive controls were: bevacizumab detected by an HRP-conjugated anti-human Fc antibody for binding to VEGF165 and VEGF121; the mouse cross-reactive VEGF-specific monoclonal antibody B20-4.1 for mouse VEGF164 and rat VEGF164; the corresponding ligand receptors human VEGFR1-Fc for human PIGF and human VEGF-B; human VEGFR2-Fc for human VEGF-C and human VEGFR2-Fc; and anti-human VEGF-D mAb for human VEGF-D. An irrelevant VHH served as a negative control.

**Inhibition of VEGF-mediated phosphorylation of ERK**

Serum-starved HUVECs were stimulated in the absence or presence of BI 836880 or bevacizumab with VEGF165 in the absence or presence of human serum albumin (HSA) for 5 minutes. Cells were fixed with 4% formaldehyde and blocked with 150 µL milk powder solution. ERK phosphorylation levels were measured by ELISA using a phospho-ERK-specific primary antibody and an anti-mouse HRP- or AP-conjugated secondary antibody.
Inhibition of VEGF-induced HUVEC proliferation

Serum-starved HUVECs were stimulated in the absence or presence of BI 836880 (0.04–10.42 nM) with 33 ng/mL VEGF165. After incubation for 3 days in the absence or presence of HSA, proliferation rates were measured by \[^{3}H\]-Thymidine incorporation. Briefly, \[^{3}H\]-Thymidine was added, and cells were incubated for 24 hours at 37°C, followed by an incubation at -20°C for 24 hours. Plates were thawed, cell fragments detached with trypsin, and \[^{3}H\]-Thymidine measured using a liquid scintillation counter (1450 Microbeta Wallac Trilux; Perkin-Elmer).

Inhibition of ANG1/2-mediated TIE2 phosphorylation

HEK293 cells transfected with human TIE2 were cultured in Poly-D-lysine-coated 96 well plates and starved for 6 hours. Cells were then stimulated in the absence or presence of BI 836880 with 2 µg/mL ANG1 or ANG2 in the absence or presence of HSA for 15 minutes. Cells were lysed and TIE2 phosphorylation levels were measured by ELISA using a plate-bound primary anti-TIE2 antibody and a secondary anti-phosphotyrosine HRP-conjugate 4G10 platinum.

Inhibition of human ANG2-mediated effects on HUVEC survival

A cellular potency assay was established based on ANG2-mediated HUVEC survival using impedance measurement on the xCELLigence real-time cell analysis (RTCA) MP instrument (Roche). HUVECs were seeded in an E-Plate 96 (Roche; 05232368001) at 25,000 cells/well (100 µL) in assay medium (EBM-2 + 10% FBS [Sigma; F7524; depleted from growth factors] + 2% gentamycin [Invitrogen; 15750]). Cells were incubated in the RTCA MP station for 24 hours, and impedance was measured every 15 minutes. After 24 hours, a dilution series of BI 836880, irrelevant VHH and 750 ng/mL recombinant human ANG2 were added. HUVECs were cultured for an additional 72 hours. Cell index values were
calculated by the RTCA software. Normalized cell index values (calculated at the time point of competitor addition) were imported in GraphPad Prism 5.0 and concentration response curves were analyzed using a non-linear regression model (log[inhibitor] vs. response).

**In vivo efficacy of BI 836880 in patient-derived xenograft models**

All animal studies were approved by the corresponding internal ethics and local government committees. Group sizes in efficacy studies were selected after performing a power analysis. Tumor xenografts used are detailed in Supplementary Table S1. Patient-derived human xenograft studies were performed at Charles River (former "Oncotest GmbH", Freiburg, Germany) and were approved by the local authorities (Regierungspräsidium Freiburg).

The tumor fragments were directly implanted into female NMRI-\(\text{Foxn}^+\text{Foxn}^+\) mice (bred and purchased from Charles River, Sulzfeld, Germany). Once tumors had reached a volume of 50–250 mm\(^3\), mice were randomized to treatment (10 mice per treatment group). Tumor-bearing mice were treated twice-weekly intraperitoneally with BI 836880 (13.7 mg/kg), bevacizumab (15 mg/kg), AMG386 (15 mg/kg) or vehicle control at (100 µL/mouse) throughout the whole experiment (2.5–9 weeks). Tumor volumes were determined twice-weekly using a caliper and body weight was measured twice-weekly as an indication of tolerability. Mice were checked daily for their well-being. Animals were sacrificed at the end of the study or when their tumors reached a volume of 1,500 mm\(^3\). Animals with necrosis at the tumor with a diameter of ≥8 mm, tumor ulceration or body weight loss of >20% were euthanized for ethical reasons. For animals that were euthanized prior to the end of the experiment, the last tumor volume measurement was carried forward until the last day of the study, provided that at least 70% of the group were still alive. Tumor growth inhibition (TGI) was calculated with the equation: \[1-((\text{treated(final)} - \text{treated (day1 treatment)})/(\text{control(final)} - \text{control(day1)})) \times 100\%\]. TGI <100% describes reduced tumor growth compared to the control group and >100% describes tumor regression.
In vivo efficacy of BI 836880 in combination with RMP 1-14 and vatalanib in LL/2 tumor model

Syngeneic experiments were performed internally and were approved by the Austrian Authority MA58 (Stadt Wien – Wasserrecht; license number GZ: 284638/2014/10). Female C57BL/6NTac mice (Bred and purchased from Taconic, Borup, Denmark) were engrafted subcutaneously with 50,000 murine LL/2 lung cancer cells diluted in 1:1 PBS and Matrigel including 5% FCS. Randomization was performed one day after cell inoculation based on body weight. Treatment was initiated on day 4 following tumor cell inoculation (10 mice per treatment group). Treatment groups received RMP 1-14 (10 mg/kg; twice-weekly intraperitoneally), BI 836880 (15 mg/kg; twice-weekly intraperitoneally), vatalanib (100 mg/kg; once-daily by oral gavage) or their combination. The control group was treated with 0.5% Natrosol (10 mL/kg; once-daily orally) and with 1×PBS (twice-weekly intraperitoneally) to reflect the same stress as in the treatment groups. The applied antibodies were formulated in 1×PBS. Vatalanib was formulated in 0.5% Natrosol. Tumor volumes were measured three times per week using a caliper. Mice were treated from day 4 until day 30 unless they were euthanized earlier due to reaching either a tumor size of 1,500 mm³ or necrosis at the tumor with a diameter of ≥8 mm. TGI was calculated as described above to compare the treatment groups.

Assessment of vessel morphology and numbers

A dedicated biomarker experiment was performed using the murine LL/2 lung cancer model. On day 14 following tumor cell injection, female mice were randomized by tumor size to treatment groups (2–3 mice per group; tumor size 81–249 mm³). Treatment groups received RMP 1-14 (10 mg/kg twice-weekly intraperitoneally), BI 836880 (15 mg/kg; twice-weekly intraperitoneally), vatalanib (100 mg/kg; once-daily by oral gavage) or their combination. The control group was treated with 0.5% Natrosol (10 mL/kg; once-daily orally) and a rat IgG2a control antibody at 10 mg/kg (twice-weekly intraperitoneally). Mice were treated for 11 days unless their tumor reached a size of 1,500 mm³ or there was necrosis at
the tumor with a diameter of ≥8 mm. On day 12, tumors were explanted, formalin-fixed and paraffin-embedded to analyze the effect of treatment on blood vessels and proximity of CD3-positive cells to vessels. Formalin-fixed, paraffin-embedded sections (2 μm) of LL/2 were stained by immunohistochemistry (IHC) for anti-CD31 / Pecam-1 (rabbit anti-CD31; Cell signaling 77699; 1:100) and anti-mouse CD3 (rabbit anti-CD3, Abcam ab16669; 1:100).

Briefly, slides were heated for 1 hour at 65°C and de-paraffinized. Antigen retrieval was performed for 20 minutes with Vector H3300 in an autoclave at 121°C/1bar. Subsequently, slides were treated for 5 minutes with 3% H2O2 diluted in PBS. Primary antibody (CD3) was incubated for 30 minutes at room temperature and conjugated with EnVision+ anti-rabbit (Dako #4003) and revealed by diaminobenzidine solution. After applying Normal Horse Serum blocking solution for 20 minutes, a second primary antibody (PECAM1 (CD31)) was incubated for 60 minutes at room temperature and conjugated with ImmPRESS-AP reagent rabbit for 30 minutes and Vector Red Substrate kit. After counterstaining with hematoxylin and mounting with coverslips, slides were scanned with a Leica Scanner and both markers were analyzed by the digital pathology software platform HALO2.0 (Indicalab®).

Regions of interest were identified through a machine-learning approach. Nuclei were detected within these regions of interest and classified as positive or negative for CD3 staining based on a threshold set as a constant across all tissue sections. CD31 staining was identified as a region of interest and transformed in annotation. Negative IgG controls were performed for each tissue section.

**Nanostring**

Total mRNA was isolated from fresh frozen tumor samples using Qiagen mRNA extraction kit and subsequently 100ng per samples were used for gene expression analysis using the nCounter Mouse PanCancer immune Profiling Panel (NanoString) according to the manufacturer’s protocol. The cartridges were scanned at most sensitive setting of 555 FOV (fields of view) with CDD camera using nCounter Digital Analyzer Version 3.1.0.1 (NanoString Technologies®, Seattle WA). For the normalization of data and for pathway
scoring the default options of nSolver Advanced Analysis Software (NanoString Technologies®) with geometric mean of the positive control probes and geometric mean of the housekeeping probes were used.

Flow Cytometry

Tumor single cell suspension were prepared using the tumor dissociation kit, mouse (Miltenyi, #130-096-730) according to manufacturer’s protocol with a gentleMACS Octo Dissociator. Red blood cells were removed by incubating the suspension with ACK lysing buffer (Thermo Fisher, #A1049201) for 1-2’ on ice and DNA was additionally digested with 100 µg/ml DNAse I (Sigma, #D4513-1VL) before staining. To exclude dead cells during gating later on, cells were stained with fixable viability stain 700 (BD, #564997). Unspecific binding to Fc receptors was blocked by incubating cells with TruStain fcX anti-mouse (clone 93, Biolegend, #101320) prior to incubation with fluorophore conjugated antibodies. Cells were stained with the following antibodies: BUV395 anti-mouse CD4 (Clone GK1.5, BD, #563790), FITC anti-mouse CD45 (Clone 30-F11, Biolegend, #103108), PE-Cy5 anti-mouse CD8a (clone 53-6.7, Biolegend, #100710), PerCP-Cy5.5 anti-mouse CD11b (clone M1/70, Biolegend, #101228), BV711 anti-mouse NKp46 (clone 29A1.4, Biolegend, #137621) and APC anti-mouse Foxp3 (clone FJK-16s, Thermo Fisher, #17-5773-82). After staining for extracellular antigens, cells were fixed and permeabilized with the Transcription Factor Buffer Set (BD, #562574) according to manufacturer’s protocol. Data acquisition was performed using a LSR II Fortessa (BD) cytometer. Doublets were excluded during gating based on FSC-H/FSC-A and SSC-H/SSC-A scatter characteristics.

Statistical analysis

To evaluate the statistical significance of tumor inhibition in PDX models, a one-tailed non-parametric Mann-Whitney-Wilcoxon U-test was performed (Sachs, 1982), based on the hypothesis that an effect would only be measurable in one direction (i.e., on the expectation of tumor inhibition but not tumor stimulation). In this study, the U-test compared the individual
TGI of two groups as measured by the absolute tumor volume on each particular day (pairwise comparisons between groups). Specifically, it was used to compare: A) the vehicle control group with each of the test groups; B) the BI 836880-treated groups with the bevacizumab- and AMG386-treated groups; and C) the groups receiving combination therapy with the groups given the respective monotherapies.

Analysis was performed on specified days, usually the last day on which at least seven mice were still alive in the comparison groups. Tumors to which the last observation carried forward methodology was applied until the day of the statistical analysis were included in the comparison. The $P$ values obtained from the U-test were adjusted using the Bonferroni-Holm correction (Holm S, 1979). By convention, $P$ values $<0.05$ indicate that the observed difference was statistically significant.

For comparison of anti-PD1 (RMP 1-14) + anti-VEGFR2 (vatalanib) treatment to BI 836880 + anti-PD1 (RMP 1-14) + anti-VEGFR2 (vatalanib) treatment, a repeated measurement mixed model (RMMM) was used to analyze log-transformed tumor volumes over time. The fixed effects Treatment, Day and the interaction term Treatment by Day were included in the statistical model. Estimated treatment differences on the log scale were back transferred to the original scale and the ratios with 0.95 confidence intervals were calculated up to day 26. Due to the exploratory nature of this analysis no adjustment of the significance level $\alpha$ was performed.

Statistical analysis of flow cytometry data was conducted using one-way ANOVA comparing the means of each treatment group with every other group and corrected for multiple comparisons (Tukey). Indication of significance: $P \leq 0.001$ (***) $P \leq 0.01$ (**), $P \leq 0.05$ (*), $P > 0.05$ (not significant and not indicated).

Statistical calculations were performed using GraphPad Prism Bioanalytic Software (version 9.3 for Windows; GraphPad Software) and SAS® (version 9.4; SAS Institute Inc.).
Results

BI 836880 selectively binds to human VEGF-A and ANG2

BI 836880 has high affinity for human VEGF165 \( (K_D = 1.4 \text{ nM}) \), human serum albumin \( (K_D = 9 \text{ nM}) \), and human ANG2 \( (K_D = 16 \text{ pM}) \). BI 836880 binds human and cynomolgus VEGF165 with similar affinities and is human/mouse/cynomolgus cross-reactive for ANG2 binding (Supplementary Table S2). BI 836880 does not bind to mouse or rat VEGF.

Binding is selective for human VEGF-A \( (EC_{50} = 1.4 \text{ nM for VEGF165 and 2.3 nM for VEGF121}) \); no binding to human VEGF-B, human VEGF-C, or human VEGF-D could be detected (Supplementary Table S2). BI 836880 shows selectivity towards ANG2 binding, as the nanobody potently inhibits and fully blocks human ANG2 binding to TIE2 with an \( IC_{50} \) of 50 pM in the presence of HSA and 45 pM in the absence of HSA. This is not the case for ANG1 binding \( (IC_{50} >2 \mu M) \). The \( IC_{50} \) for the positive control, AMG386, against ANG1/TIE 2 is 25 nM \((\pm 7 \text{ nM})\), confirming the cross-reactivity of AMG386 to ANG1 (Coxon et al., 2010).

BI 836880 inhibits signaling downstream of VEGF and ANG2 and inhibits endothelial cell proliferation and survival

VEGF binding to its receptors leads to the activation of several intracellular signaling pathways, including the phosphorylation of ERK. Dose dependent ERK phosphorylation can readily be detected in HUVECs upon VEGF stimulation (Fig 1A). Treatment of VEGF-stimulated HUVECs with BI 836880 resulted in inhibition of ERK phosphorylation by 93 ± 2% with \( IC_{50} \) at 1.1 ± 0.3 nM in the absence of HSA (Fig. 1B) and by 84 ± 5% in the presence of HSA with \( IC_{50} \) at a concentration of 1.4 ± 0.2 nM (data not shown). In comparison, bevacizumab showed 99 ± 1% inhibition of ERK phosphorylation with \( IC_{50} \) at 1.6 ± 0.3 nM in the absence of HSA (Fig 1B) and by 94 ± 3% in the presence of HSA with \( IC_{50} \) 1.7 ± 0.1 nM (data not shown). In addition, VEGF-A acts as an endothelial cell-specific mitogen. BI 836880 potently inhibited VEGF-induced HUVEC proliferation by 84 ± 8% with an \( IC_{50} \) of 0.8
± 0.14 nM in the absence of HSA (Fig. 1C) and by 93 ± 5% in the presence of HSA, with an IC₅₀ of 0.6 ± 0.09 nM (data not shown).

Binding of the ligands ANG2 or ANG1 to the TIE2 receptor results in receptor phosphorylation. BI 836880 fully inhibited ANG2-induced TIE2 phosphorylation in the presence of HSA (IC₅₀ 3.2 ± 2.3 nM) as well as in the absence of HSA (IC₅₀ 1.3 ± 1.3 nM) in HEK293 cells overexpressing human TIE2 (Fig. 2A). However, TIE2 phosphorylation induced by ANG1 was not inhibited by BI 836880 (Fig. 2B). ANG2 is a pro-survival factor for endothelial cells, and BI 836880 completely inhibited ANG2-induced survival of HUVECs (IC₅₀ 1.9 ± 0.4 nM; Fig. 2C).

**BI 836880 shows in vivo efficacy in several patient-derived xenograft models**

BI 836880 exhibited significant antitumor efficacy in a range of patient-derived xenograft models, including pancreatic cancer (PAXF 546, PAXF 736, and PAXF 1872), non-small cell lung cancer (LXFE 211 and LXFE 1422), renal cell cancer (RXF 631 and RXF 1220), breast cancer (MAXF 401 and MAXF 1322), ovarian cancer (OVXF 1353) and colon cancer (CXF 243; Table 1 and Supplementary Table S1). TGI following BI 836880 treatment ranged from 51% (RXF 1220) to 92% (MAXF 401). In general, no loss of body weight or deaths were observed. Exceptions were the cachexia-inducing tumor xenograft model RXF 1220 and the LXFE211 model, for which control and bevacizumab treatment led to reduced body weights (Supplementary Figure 1). BI 836880 showed significantly greater TGI compared with inhibition of VEGF-A alone (bevacizumab) in seven out of 11 tumor xenograft models tested (PAXF 546, PAXF 736, PAXF 1872, LXFE 211, LXFE 1422, RXF 1220, MAXF 1322; Table 1). TGI following BI 836880 treatment was significantly greater than that observed with the ANG1/ANG2-inhibiting molecule AMG386 in two models (PAXF 546, LXFE 1422; Table 1 and Fig. 3) and showed a trend towards increased TGI in several other models (Table 1).
Combining VEGF/ANG2 inhibition with PD-1 inhibition improves in vivo antitumor efficacy

To test the combination of BI 836880 with an anti-PD-1 in a syngeneic mouse tumor model with a functional immune system we used BI 836880 as an ANG2 antagonist; however, it could not be used as a VEGF inhibitor because of its low cross-reactivity to mouse VEGF. Due to the toxicities observed upon administering three antibodies in combination (murine anti-VEGF antibody B20, anti-PD-1 antibody RMP 1-14, and BI 836880) we replaced the anti-VEGF antibody with vatalanib, the most specific VEGFR2 small-molecule inhibitor available (Wood et al., 2000). While vatalanib may be the most specific VEGFR2 inhibitor available, it is still a multi-kinase inhibitor, with the potential to inhibit other VEGF receptors (VEGFR1 and VEGFR3), as well as platelet-derived growth factor receptor and c-Kit. In the LL/2 syngeneic tumor model, treatment with RMP 1-14 (murine anti-PD-1 monoclonal antibody), BI 836880 (ANG2 inhibitor), and vatalanib (VEGFR2 inhibitor) alone resulted in TGI values of 22%, 27%, and 43%, respectively (Fig. 4A–C). Combination of RMP 1-14 with vatalanib resulted in a significantly increased antitumor effect compared with either monotherapy, with TGI of 73% (P = 0.0056 for RMP 1-14 vs. RMP 1-14 + vatalanib and P = 0.0056 for vatalanib vs. RMP 1-14 + vatalanib; Fig. 4D). Combination of RMP 1-14 with BI 836880 (ANG2 inhibition only) resulted in a TGI of 48% compared to control (Fig. 4E). Similarly, combining RMP 1-14 with vatalanib increased the time until tumors were larger than 500 mm³ (Fig. 4G). Addition of BI 836880 (anti-ANG2) did not significantly improve TGI vs. RMP 1-14 + vatalanib (80% vs. 73%; P = 0.138; Fig. 4F); however, on day 26, tumor volumes were reduced by 17% compared to treatment with vatalanib + RMP 1-14 (P = 0.056, one-sided), indicative of an additional benefit from ANG2 inhibition (Supplementary Table S3). In addition, there was a further increase in time for tumors to reach a size of 500 mm³ (Fig. 4G).
Combining BI 836880 with PD-1 inhibition affects vessel morphology and reduces endothelial vessel numbers

IHC analysis showed that the combination of VEGFR2 and ANG2 inhibition, as well as the triple combination of ANG2, VEGFR2, and PD-1 inhibition, had a strong effect on vessel morphology (Fig. 5A). Morphological assessment of CD31 expression on endothelial cells showed a reduction of endothelial immature vessel numbers with ANG2 and VEGFR inhibition (with or without PD-1 inhibition) versus control (Fig. 5B). In addition, we observed a trend towards an increase of CD3-positive T cell density after combined VEGFR2, ANG2 and PD-1 inhibition (Fig. 5C). mRNA expression analysis using the nCounter Mouse PanCancer immune Profiling Panel (NanoString) showed a decrease of CD31 expression after combined inhibition of ANG2 and VEGFR (with or without PD-1 inhibition (Fig. 5D). Pathway scoring using nSolver Advances Analysis Software (NanoString) (log2) detected the chemokine (n=85 genes) and cytokine receptor (n=192 genes) pathway as highly increased following ANG2 and VEGFR inhibition, with or without PD-1 inhibition (Fig. 5E-F). Ten genes (Cxcl2, Cxcl3, Cxcr2, Lcn2, S100a8, Cd276, Cx3cr1, Cxcl9, Itga4 and Ly86) were found to be the most significantly deregulated genes. To elucidate treatment effects on tumor infiltrating T lymphocyte subsets in more detail, LL/2 tumor cell suspensions were analyzed by flow cytometry. Inhibition of PD-1 led to a ~3-fold increase of CD8a+ cells among all T cells, whereas the increase due to inhibition of VEGFR2 and the combined inhibition of PD-1, VEGFR2 and ANG2 was ~2-fold. Effects on the proportion of CD4+ T cells were less pronounced across all treatment groups (Fig. 6A and 6B). The ratio of CD8a+/CD4+ among all infiltrating immune cells confirmed the shift in the T cell composition towards an increase in CD8a+ cells (Fig. 6C). Importantly, the relative abundance of Foxp3+ regulatory T cells among all CD4+ T cells was stable across all treatment groups (Fig. 6D).
Discussion

In vitro, BI 836880 potently and selectively neutralized VEGF-A. BI 836880 blocked ERK phosphorylation, indicating inhibition of VEGF downstream signaling, and impaired HUVEC proliferation. BI 836880 also inhibited signaling downstream of ANG2 receptor binding and stimulation, leading to a decrease in endothelial cell survival. Readouts were chosen to best reflect the physiological roles of VEGF and ANG2 in endothelial cells, with a proliferation readout for VEGF and a survival readout for ANG2. In addition, ANG2 signaling was investigated using HEK293 cells overexpressing Tie2. ANG2 treatment of these cells resulted in increased phosphorylation of Tie2, indicating an agonistic effect of ANG2. ANG2 is mainly described as an ANG1 antagonist (Gerald et al., 2013), however, ANG2 can act as partial Tie2 agonist under certain conditions (Gale et al., 2002; Coffelt et al., 2010). Recent data suggest that ANG2 might act as Tie2 agonist in the absence of Tie1 (Seegar et al., 2010). In HEK293 cells overexpressing Tie2 we have shifted the balance between Tie1 and Tie2 and this might favor Tie2 agonism.

In vivo, BI 836880 inhibited angiogenesis and showed superior activity to bevacizumab or AMG386 alone in a broad range of patient-derived xenograft models. TGI by BI 836880 was significantly stronger in seven of the eleven models tested compared with VEGF inhibition alone. This demonstrates that addition of ANG2 inhibition to interruption of the VEGF pathway in these murine tumor models has a potential therapeutic advantage. A study by Schiffmann et al (2015) showed that dual targeting of VEGF and ANG2 resulted in increased vascular normalization compared to single agent treatment, for instance in terms of vessel leakiness and hypoxia. The authors also observed improved endothelial integrity, such as increased pericyte coverage and reduced permeability.

In our study, TGI by BI 836880 varied between the patient-derived xenograft models with values between 92% and 51%. There was no correlation based on cancer type or genotype. Reasons for the differences between models could be due to differences in vessel density, the ratio between mature and immature vessels, or in the sensitivity of the tumors to tolerate
hypoxia. Interestingly, Schiffmann et al observed a correlation between the presence of CD177+ neutrophils and reduced survival of CRC patients after bevacizumab treatment (Schiffmann et al., 2019). Treatment of the VEGF resistant LLC mouse tumor model with BI 836880 and an antibody against murine VEGF (B20) led to tumor growth inhibition. Similar results were obtained by VEGF inhibition combined with depletion of tumor-infiltrating neutrophils. This indicates that neutrophils might mediate VEGF resistance, and that BI 836880 treatment might be able to restore sensitivity towards VEGF inhibition. Thus, it is possible that the differences in sensitivity towards BI 836880 might be linked to differences in neutrophil content of the tumors.

Our findings are consistent with those observed with the bispecific VEGF-A/ANG2 CrossMab antibody, vanucizumab, which has demonstrated strong antitumor efficacy in human tumor xenografts and a chemo-resistant colorectal carcinoma model, as well as inhibition of angiogenesis and enhanced vessel maturation (Kienast et al., 2013; Mueller et al., 2019). While these studies demonstrate the potential value of combined VEGF/ANG2 inhibition, they have not yet been translated into clinical success. Vanucizumab monotherapy showed evidence of antitumor activity in a phase I study of patients with advanced solid tumors (Hidalgo et al., 2018). However, in the phase II McCAVE study, it did not improve efficacy versus bevacizumab when combined with modified FOLFOX-6 in patients with metastatic colorectal carcinoma (Bendell et al., 2020). It remains to be seen whether the smaller size of the BI 836880 nanobody could offer advantages over larger full-size antibodies, such as vanucizumab, particularly in facilitating greater tumor penetration and drug availability in the target tissue.

Reflecting the relative novelty of the technology, only a few therapeutic nanobodies have entered clinical trials to date. Caplacizumab, an anti-von Willebrand factor nanobody® showed promise for the treatment of acquired thrombotic thrombocytopenic purpura in a double-blind, controlled trial (Scully et al., 2019). In addition, vobarilizumab, an anti-interleukin-6 receptor nanobody®, showed promising results in a phase I/II study for the
treatment of rheumatoid arthritis (Kim et al., 2015). This suggests that nanobody® molecules are suitable for use in humans and are able to modify human disease.

As hypothesized, our results showed that combining PD-1 inhibition with VEGF inhibition resulted in superior antitumor activity in an LL/2 tumor model compared with inhibition of either pathway alone. VEGF/ANG2 inhibition, with or without PD-1 inhibition, also had a strong effect on vessel morphology and reduced vessel numbers compared with control treatment. This is consistent with other preclinical data showing that blockade of PD-1 and VEGFR2 significantly inhibited tumor growth compared with each monoclonal antibody alone (Yasuda et al., 2013). In this murine cancer model, VEGFR2 blockade inhibited tumor microvessel development while PD-1 blockade enhanced T cell recruitment into tumors and activated local immunity. The authors hypothesized that anti-angiogenic treatment enabled normalization of tumor vessels, thus restoring blood flow and improving transportation of anti-cancer drugs (anti-PD-1) to the tumors (Yasuda et al., 2013). Furthermore, PD-L1 has previously been shown to be upregulated during anti-VEGF treatment and combined VEGFR2/PD-L1 inhibition was associated with improved tumor response and higher T-cell infiltration compared with VEGFR2 and PD-L1 inhibition alone (Allen et al., 2017).

We hypothesized that addition of ANG2 inhibition to VEGF and PD-1 inhibition could further improve antitumor activity in vivo by improving vessel normalization and thus facilitating the delivery of anti-PD-1 antibodies to the tumor as well as removing potential barriers to effector T cells. BI 836880 has previously been shown to reduce micro-vessel density and intra-tumoral hypoxia in tumor xenografts (Schiffmann et al., 2019). Our data showed a trend towards vessel normalization, reduced tumor volumes, as well as an increase in time for tumors to reach a size of 500 mm³ for the triple combination versus anti-PD-1 plus anti-VEGFR2 inhibition. Evaluation of CD3-positive T cells also showed a trend towards an increased density of this cell population after treatment with the triple combination, compared to single and double agent treatments. Within the tumor infiltrating T cell lymphocyte pool, we observed a favorable shift towards increased proportions of CD8α+ T cells in treatment groups of PD-1 and VEGFR2 single inhibition and in the combination
group with PD-1, VEGFR2 and ANG2 inhibition. We hypothesize that the efficacy is driven by improved infiltration of CD3-positive T cells into the tumor and an augmented anti-tumor immunity due to an increased CD8a^+ T lymphocyte fraction.

BI 836880 has low cross-reactivity to mouse VEGF. We therefore chose vatalanib as VEGFR2 inhibitor in the syngeneic LL/2 tumor model. Vatalanib has strong inhibitory activity towards VEGFR2 (IC_{50} value of 37 nM) and VEGFR1 (IC_{50} value of 77 nM). BI 836880 targets VEGF-A, the ligand of VEGFR1 and VEGFR2. Activity of vatalanib towards additional targets is lower, with inhibition of FLK1 with an IC_{50} of 270 nM, VEGFR3 with an IC_{50} of 660 nM, PDGFR with an IC_{50} of 580 nM and c-Kit with an IC_{50} of 730 nM. One additional point to consider is that these experiments were conducted in only one tumor model: the LL/2 syngeneic tumor model. It is possible that evaluating other tumor models with different biology may better elucidate the relative contribution of VEGF and ANG2 signaling in combination with anti-PD-1 treatment. To this end, a combination of BI 836880 and ezabenlimab (BI 745091, an anti PD-1 inhibitor) is currently under clinical investigation in different tumor types (NCT03468426, NCT03697304, and NCT03972150). Promising clinical activity was observed in patients with HCC (complete response in 1/29 patients and partial response in 11/29 patients), SCLC (partial response in 5/26 patients) and GBM (partial response in 6/30 patients) (Girard et al., 2021).

Clinical data support the combination of VEGF blockade with immune checkpoint inhibition, with clinical benefit observed in several tumor types. For example, a phase III trial showed that the combination of atezolizumab and bevacizumab resulted in prolonged overall survival (OS) and progression-free survival (PFS) versus sorafenib in patients with unresectable hepatocellular carcinoma (Finn et al., 2020). The addition of atezolizumab to bevacizumab and chemotherapy also significantly improved OS and PFS as first-line treatment of metastatic non-squamous NSCLC (Socinski et al., 2018). Addition of VEGF/ANG2 inhibition to PD-L1 inhibition has also been assessed clinically. The combination of vanucizumab with atezolizumab was evaluated in a single-arm extension of a phase I study in 17 patients with platinum-resistant recurrent ovarian cancer; however,
results from this small study did not suggest any benefits from combining VEGF/ANG2 inhibition with PD-L1 blockade in this indication (Oaknin et al., 2017).

In summary, BI 836880 is a novel agent which selectively and potently binds to VEGF and ANG2. We showed that combining VEGF and ANG2 inhibition resulted in improved antitumor activity versus inhibition of either pathway alone. Our preclinical data also supports the rationale for the combination of VEGF/ANG2 inhibition with an anti-PD-1 antibody. BI 836880 is currently under investigation in several phase I and phase II clinical trials as monotherapy (NCT02674152 and NCT02689505) and in combination with ezabenlimab (NCT03468426, NCT03697304, and NCT03972150).
Acknowledgments

We acknowledge the contribution of Dietmar Zellner, who was involved in data analysis and contributed to the development of the first drafts of this paper but sadly passed away prior to submission. We also acknowledge the contributions of Sarah Müllner, Doris Zalusky, Florian Martin, Rebecca Langlois, Alexander Wlachos, Astrid Jeschko and Thomas Madensky who performed the in vivo and in vitro studies.

Medical writing support for the development of this manuscript, under the direction of the authors, was provided by Caroline Allinson, of Ashfield MedComms, an Ashfield Health company, and funded by Boehringer Ingelheim.

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References

Allen E, Jabouille A, Rivera LB, Lodewijckx I, Missiaen R, Steri V, Feyen K, Tawney J, Hanahan D, Michael IP and Bergers G (2017) Combined antiangiogenic and anti-PD-L1 therapy stimulates tumor immunity through HEV formation. Science translational medicine 9:eaak9679.

Bendell JC, Sauri T, Gracián AC, Alvarez R, López-López C, García-Alfonso P, Hussein M, Miron ML, Cervantes A, Montagut C, Vivas CS, Bessudo A, Plezia P, Moons V, Andel J, Bennouna J, van der Westhuizen A, Samuel L, Rossomanno S, Boetsch C, Lahr A, Franjkovic I, Heil F, Lechner K, Krieter O and Hurwitz H (2020) The MCeAVE Trial: Vanucizumab plus mFOLFOX-6 Versus Bevacizumab plus mFOLFOX-6 in Patients with Previously Untreated Metastatic Colorectal Carcinoma (mCRC). The oncologist 25:e451-e459.

Brown JL, Cao ZA, Pinzon-Ortiz M, Kendrew J, Reimer C, Wen S, Zhou JQ, Tabrizi M, Emery S, McDermott B, Pablo L, McCoon P, Bedian V and Blakey DC (2010) A human monoclonal anti-ANG2 antibody leads to broad antitumor activity in combination with VEGF inhibitors and chemotherapy agents in preclinical models. Molecular cancer therapeutics 9:145-156.

Carmeliet P and Jain RK (2011) Principles and mechanisms of vessel normalization for cancer and other angiogenic diseases. Nature reviews Drug discovery 10:417-427.

Coffelt SB, Tal AO, De Palma M, Patel S, Urbich C, Biswas SK, Murdoch C, Plate KH, Reiss Y and Lewis CE (2010) Angiopoietin-2 regulates gene expression in TIE2-expressing monocytes and augments their inherent proangiogenic functions. Cancer research 70:5270-5280.

Coxon A, Bready J, Min H, Kaufman S, Leal J, Yu D, Lee TA, Sun JR, Estrada J, Bolon B, McCabe J, Wang L, Rex K, Caenepeel S, Hughes P, Cordover D, Kim H, Han SJ, Michaels ML, Hsu E, Shimamoto G, Cattley R, Hurh E, Nguyen L, Wang SX, Ndfor A, Hayward U, Falcón BL, McDonald DM, Li L, Boone T, Kendall R, Radinsky R and Oliner JD (2010) Context-dependent role of angiopoietin-1 inhibition in the suppression of angiogenesis and tumor growth: implications for AMG 386, an angiopoietin-1/2-neutralizing peptibody. Molecular cancer therapeutics 9:2641-2651.

Fiedler U and Augustin HG (2006) Angiopoietins: a link between angiogenesis and inflammation. Trends in immunology 27:552-558.

Finn RS, Qin S, Ikeda M, Galle PR, Ducrèux M, Kim TY, Kudo M, Breder V, Merle P, Kaseb AO, Li D, Verret W, Xu DZ, Hernandez S, Liu J, Huang C, Mulla S, Wang Y, Lim HY, Zhu AX and Cheng AL (2020) Atezolizumab plus bevacizumab in unresectable hepatocellular carcinoma. The New England journal of medicine 382:1894-1905.

Gale NW, Thurston G, Hackett SF, Renard R, Wang Q, McClain J, Martin C, Witte C, Witte MH, Jackson D, Suri C, Campochiaro PA, Wiegand SJ and Yancopoulos GD (2002) Angiopoietin-2 Is Required for Postnatal Angiogenesis and Lymphatic Patterning, and Only the Latter Role Is Rescued by Angiopoietin-1. Developmental Cell 3:411-423.

Gerald D, Chintharlapalli S, Augustin HG and Benjamin LE (2013) Angiopoietin-2: an attractive target for improved antiangiogenic tumor therapy. Cancer research 73:1649-1657.

Girard N, Wermke M, Ledin E, Kim DW, Ghiringhelli F, Bennouna J, Lesimple T, Felip E, Berz D, Lee JS, Jeanson A, Mascaux C, Voskoboynik M, Serwatowski P, Burger MC, Landsteiner HT, Chen V, Jayadeva G, Alt J and Hackanson B (2021) 532P Phase Ib study evaluating BI 836880 (VEGF/Ang2 nanobody) in combination with ezabenlimab (BI 754091; anti-PD-1 antibody) in patients with solid tumours. Annals of Oncology 32:S597.

Hashizume H, Falcón BL, Kuroda T, Baluk P, Coxon A, Yu D, Bready JV, Oliner JD and McDonald DM (2010) Complementary actions of inhibitors of angiopoietin-2 and VEGF on tumor angiogenesis and growth. Cancer research 70:2213-2223.

Hidalgo M, Martinez-Garcia M, Le Tourneau C, Massard C, Garralda E, Boni V, Taus A, Albanell J, Sablin MP, Alt M, Bahleda R, Varga A, Boetsch C, Franjkovic I, Heil F, Lahr A, Lechner K, Morel A, Nayak T, Rossomanno S, Smart K, Stubenrauch K and Krieter O (2018) First-in-human Phase I study of single-agent vanucizumab, a first-in-class bispecific anti-angiopoietin-2/anti-VEGF-A antibody, in adult patients with advanced solid tumors. Clinical cancer research : an official journal of the American Association for Cancer Research 24:1536-1545.
Holm S (1979) A simple sequentially rejective multiple test procedure. *Scan J Stat* **6**:65-70.

Kienast Y, Klein C, Scheuer W, Raemsch R, Lorenzon E, Bernicke D, Herting F, Yu S, The HH, Martarello L, Gassner C, Stubenrauch KG, Munro K, Augustin HG and Thomas M (2013) Ang-2-VEGF-A CrossMab, a novel bispecific human IgG1 antibody blocking VEGF-A and Ang-2 functions simultaneously, mediates potent antitumor, antiangiogenic, and antimetastatic efficacy. *Clinical cancer research : an official journal of the American Association for Cancer Research* **19**:6730-6740.

Kim GW, Lee NR, Pi RH, Lim YS, Lee YM, Lee JM, Jeong HS and Chung SH (2015) IL-6 inhibitors for treatment of rheumatoid arthritis: past, present, and future. *Archives of pharmacal research* **38**:575-584.

Lanitis E, Irving M and Coukos G (2015) Targeting the tumor vasculature to enhance T cell activity. *Current opinion in immunology* **33**:55-63.

Motz GT, Santoro SP, Wang LP, Garrabrants T, Lastra RR, Hagemann IS, Lal P, Feldman MD, Benencia F and Coukos G (2014) Tumor endothelium FasL establishes a selective immune barrier promoting tolerance in tumors. *Nature medicine* **20**:607-615.

Mueller T, Freystein J, Lucas H and Schmoll HJ (2019) Efficacy of a bispecific antibody co-targeting VEGFA and Ang-2 in combination with chemotherapy in a chemoresistant colorectal carcinoma xenograft model. *Molecules (Basel, Switzerland)* **24**:2865.

Oaknin A, Vergote I, Ray-Coquard I, Leary A, Rodriguez Freixinos V, Concin N, Toussaent P, Massard C, Fariñas-Madrid L, Van Nieuwenhuysen E, Lahr A, Franjkovic I, Rossmanno S, Gerber P, Nayak T, Heil F, Boetsch C, Sahbi A, Longauer K and Krieter O (2017) Vanucizumab (VAN) in combination with atezolizumab (ATEZO) for platinum-resistant recurrent ovarian cancer (PROC): results from a single arm extension phase of the phase I study BP28179. *Annals of Oncology* **28**:v335.

Reginato S, Gianni-Barrera R and Banfi A (2011) Taming of the wild vessel: promoting vessel stabilization for safe therapeutic angiogenesis. *Biochemical Society transactions* **39**:1654-1658.

Sachs L (1982) Angewandte Statistik. 8th ed., in *Section 394 Der Vergleich zweier unabhängiger Stichproben: U-Test nach Wilcoxon, Mann und Whitney*, Springer-Verlag Berlin.

Schiffmann LM, Fritsch M, Gebauer F, Günther SD, Stair NR, Seeger JM, Thangarajah F, Dieplinger G, Bludau M, Alakus H, Göbel H, Quaas A, Zander T, Hilberg F, Bruns CJ, Kashkar H and Coutelle O (2019) Tumour-infiltrating neutrophils counteract anti-VEGF therapy in metastatic colorectal cancer. *British journal of cancer* **120**:69-78.

Scully M, Cataland SR, Peyvandi F, Coppo P, Knöbl P, Kremer Hovinga JA, Metjjan A, de la Rubia J, Pavenksi K, Callewaert F, Biswas D, De Winter H and Zeldin RK (2019) Caplacizumab treatment for acquired thrombotic thrombocytopenic purpura. *New Engl J Med* **380**:335-346.

Seegar TC, Eller B, Tzvetkova-Robev D, Kolev MV, Henderson SC, Nikolov DB and Barton WA (2010) Tie1-Tie2 interactions mediate functional differences between angiopoietin ligands. *Molecular cell* **37**:643-655.

Socinski MA, Jotte RM, Cappuzzo F, Orlandi F, Stroyakovskiy D, Nogami N, Rodriguez-Abreu D, Moro-Sibilot D, Thomas CA, Barlesi F, Finley G, Kelsch C, Lee A, Coleman S, Deng Y, Shen Y, Kowanetz M, Lopez-Chavez A, Sandler A and Reck M (2018) Atezolizumab for first-line treatment of metastatic nonsquamous NSCLC. *New Engl J Med* **378**:2288-2301.

Vergote I, Scambia G, O'Malley DM, Van Calster B, Del Campo JM, Meier W, Biamis A, Colombo N, Wenham RM, Covens A, March C, Raza Mirza M, Kroep JR, Ma H, Pickett CA and Monk BJ (2019) Trebananib or placebo plus carboplatin and paclitaxel as first-line treatment for advanced ovarian cancer (TRINOVA-3/ENGOT-ov2/GOG-3001): a randomised, double-blind, phase 3 trial. *The Lancet Oncology* **20**:862-876.

Wood JM, Bold G, Buchdunger E, Cozens R, Ferrari S, Frei J, Hofmann F, Mestan J, Matt H, O'Reilly T, Persohn E, Rösel J, Schnell C, Stover D, Theuer A, Towbin H, Wenger F, Woods-Cook K, Menrad A, Siemeister G, Schirner M, Thierauch KH, Schneider MR, Drevs J, Martiny-Baron G and Totzek F (2000) PTK787/ZK 222584, a novel and potent inhibitor of vascular endothelial...
growth factor receptor tyrosine kinases, impairs vascular endothelial growth factor-induced responses and tumor growth after oral administration. *Cancer research* **60**:2178-2189.

Yang J, Yan J and Liu B (2018) Targeting VEGF/VEGFR to modulate antitumor immunity. *Frontiers in immunology* **9**:978.

Yasuda S, Sho M, Yamato I, Yoshiji H, Wakatsuki K, Nishiwada S, Yagita H and Nakajima Y (2013) Simultaneous blockade of programmed death 1 and vascular endothelial growth factor receptor 2 (VEGFR2) induces synergistic anti-tumour effect in vivo. *Clinical and experimental immunology* **172**:500-506.

Zinzalla V, Drobits-Handl B, Savchenko A, Rinnenthal J, Bauer MJ, Sanderson M, Blake SM, Schweifer N, Vries RGJ, Clevers H and Kraut N (2019) BI 905677: a first-in-class LRP5/6 antagonist targeting Wnt-driven proliferation and immune escape. *Molecular cancer therapeutics* **79**(Suppl13):AbstrDDT0101.

Footnotes

Funding

This study was funded by Boehringer Ingelheim.

Authors’ Disclosures

I. Hofmann, A. Baum, M.H. Hofmann, A. Gschwind, F. Trapani, C. Reichel-Voda, D. Ehrensperger, N. Schweifer, M. Aichinger, F. Ebner, N. Budano, M. Sykora, N. Kraut, F. Hilberg and K.-P. Künkele are or were employees of Boehringer Ingelheim at the time of the study. E. Depla, and J. Boucneau are or were employees of Ablynx NV at the time of the study. M.H. Hofmann, F. Hilberg, J. Boucneau, and E. Depla are inventors on a patent licensed to Boehringer Ingelheim describing generation and use of VEGF/ANG2 nanobodies and/or their combination with PD-1.
Legends for Figures

Figure 1.

BI 836880 inhibits VEGF signaling and endothelial cell proliferation. A, Serum-starved HUVECs were stimulated with VEGF165 (0 – 100 ng/mL) for 5 minutes. Cells were fixed and phospho-ERK was measured by ELISA using an anti-HRP secondary antibody. VEGF165 treatment led to a dose dependent increase of ERK phosphorylation. The data shown represents mean ± SEM of three biological replicates. B, Serum-starved HUVECs were stimulated in the absence or presence of BI 836880 (0.16–20 nM) or bevacizumab (0.63 – 20 nM) with VEGF165 (10 ng/mL) for 5 minutes in the absence of HSA. Cells were fixed and phospho-ERK was measured by ELISA using an anti-AP secondary antibody. The data shown represents mean ± SEM of two biological replicates. BI 836880 and bevacizumab inhibited ERK phosphorylation. C, Serum-starved HUVECs were stimulated in the absence or presence of BI 836880 (0.04–10.42 nM) with VEGF165 (33 ng/mL) in the absence of HSA for 3 days. The data shown represents mean ± SEM of two biological replicates. BI 836880 inhibited VEGF-induced HUVEC proliferation. RLU, relative light units.

Figure 2.

BI 836880 inhibits ANG2 but not ANG1 signaling. A, HEK293 cells transfected with human TIE2 were stimulated in the absence or presence of BI 836880 with 2 μg/mL ANG2 in the absence of HSA for 15 minutes. The data shown represents mean ± SEM of two biological replicates. BI 836880 inhibited ANG2-induced TIE2 phosphorylation. B, HEK293 cells transfected with human TIE2 were stimulated in the absence or presence of BI 836880 with 2 μg/mL ANG1 in the absence of HSA for 15 minutes. The data shown represents mean ± SEM of two biological replicates. BI 836880 did not inhibit ANG1-induced TIE2 phosphorylation levels. C, HUVECs were incubated with a dilution series of BI 836880, an irrelevant VHH, and recombinant human ANG2 (750 ng/mL). BI 836880 inhibited ANG2-induced HUVEC survival. hANG2, human ANG2; pTIE2, TIE2 phosphorylation.
Figure 3.

BI 836880 induces tumor growth delay in patient-derived xenograft models. Female NMRI-Foxn1nu mice bearing PAXF 546 (pancreatic cancer; A) or LXFE 1422 (NSCLC; B) tumors were treated twice-weekly with BI 836880 (13.7 mg/kg), bevacizumab (15 mg/kg), AMG 386 (15 mg/kg) or vehicle control (100 μL/mouse) for 2.5–9 weeks. Tumor volumes were determined twice-weekly. Average tumor volumes are shown (±SEM).

Figure 4.

Combining VEGF/ANG2 inhibition with PD-1 inhibition improves in vivo efficacy. Female C57BL/6NTac mice bearing LL/2 lung cancer tumors were treated with RMP 1-14 (anti-PD-1 antibody; 10 mg/kg twice-weekly intraperitoneally), vatalanib (anti-VEGFR2 small molecule inhibitor; 100 mg/kg; once-daily by oral gavage), BI 836880 (used as anti-mouse ANG2 nanobody; 15 mg/kg; twice-weekly intraperitoneally) or their combination. The control group was treated with 0.5% Natrosol (10 mL/kg; once-daily orally) and with 1×PBS (twice-weekly intraperitoneally). Mice (10 per treatment group) were treated from day 4 following tumor cell inoculation until day 30. Tumor volumes were determined three times per week and are shown for each animal (A–F). Panel G shows a Kaplan–Meier curve for tumors reaching a volume of >500 m3 by treatment group. *Significantly different to TGI with anti-PD-1 alone (P = 0.0056) and with anti-VEGFR2 alone (P = 0.0056).

Figure 5.

ANG2 and VEGFR2 inhibition induces a reduction in endothelial vessel numbers. Formalin-fixed, paraffin-embedded sections of LL/2 tumors from mice treated in a dedicated biomarker experiment for 11 days with RMP 1-14 (anti-PD-1), vatalanib (anti-VEGFR2), and BI 836880 (anti-ANG2) were stained by immunohistochemistry for anti-PECAm-1 (anti-CD31; A). Endothelial vessel numbers were reduced with ANG2 and VEGFR2 inhibition (with or without PD-1 inhibition); shown are mean vessel numbers ± standard deviation (B). CD3-positive T cell density was increased after combined VEGFR2, ANG2 and PD-1 inhibition; shown is T
cell density of replicates as well as mean T cell density ± standard deviation (C). Tumor samples were analyzed for CD31 mRNA (nCounter, NanoString). Shown is mean ± standard deviation (D). Tumor samples were analyzed in nSolver (NanoString) for chemokine (E) or cytokine pathway (F).

**Figure 6.**

PD-1 and VEGFR2 inhibition change tumor infiltrating T lymphocyte composition. Tumor single cell suspensions derived from mice treated for 11 days with indicated combinations of RMP 1-14 (anti-PD-1), vatalanib (anti-VEGFR2) and BI 836880 (anti-ANG2) were analyzed by flow cytometry. Representative flow plots of CD4⁺ and CD8a⁺ T cells (sub-gated for living CD45⁺CD11b⁻NKp46⁻Thy1.2⁺). Numbers in the respective gates show frequency of parent population (A). Plots showing frequencies of CD8a⁺ (left) and CD4⁺ (right) T cells as percent of parent population (B). Ratio of fraction of CD8a⁺/CD4⁺ of CD45⁺ cells (C). Foxp3⁺ regulatory T cells as percentage of all CD4⁺ T cells (D). (B), (C) and (D) show individual samples whereas bar and plunger indicate mean + SD. n=6 in each treatment group, samples with less than 30 events recorded in the CD8a⁺ gate were excluded from analysis.
Table 1. *In vivo* efficacy of BI 836880, bevacizumab, and AMG386 in patient-derived xenograft models. Patient-derived human xenografts (genotype information in Supplementary Table S1) were directly implanted into female NMRI-\textit{Foxn1} \textsuperscript{nu} mice. Once tumors had reached a volume of 50–250 mm\textsuperscript{3}, mice (10 per treatment group) were randomized to twice-weekly treatment with BI 836880 (13.7 mg/kg), bevacizumab (15 mg/kg), AMG386 (15 mg/kg), or vehicle control (100 μL/mouse) for 2.5–9 weeks. Tumor volumes were determined twice-weekly. A one-tailed non-parametric Mann-Whitney-Wilcoxon U-test was performed to compare tumor growth inhibition. *P* values were adjusted using the Bonferroni-Holm correction; *P* values ≤0.05 were considered statistically significant (in bold).

| Indication | Model   | Bevacizumab | AMG386 | BI 836880 | *P* value |
|------------|---------|-------------|--------|-----------|-----------|
|            |         |             |        | BI 836880 vs. Bevacizumab | BI 836880 vs. AMG386 |
| Pancreas   | PAXF 546| 59          | 50     | 87        | 0.0216    | 0.0114    |
|            | PAXF 736| 25          | 52     | 64        | 0.0044    | 0.2345    |
|            | PAXF 1872| 13         | 62     | 57        | 0.0105    | 1.0000    |
| Lung       | LXFE 211| 37          | 58     | 72        | 0.0004    | 0.0716    |
|            | LXFE 1422| 63         | 64     | 84        | 0.0146    | 0.0146    |
| Renal      | RXF 631 | 51          | 50     | 63        | 0.2176    | 0.2176    |
|            | RXF 1220| 10          | 20     | 51        | 0.0304    | 0.1388    |
| Tissue  | Type  | Measurement | Measurement  | Measurement | Measurement |
|---------|-------|-------------|--------------|-------------|-------------|
| Breast  | MAXF 401 | 68          | 54           | 92          | 0.0630      | 0.0630      |
|         | MAXF 1322 | 31          | 52           | 70          | 0.0090      | 0.3697      |
| Ovary   | OVXF 1353 | 47          | 65           | 71          | 0.3526      | 0.3526      |
| Colon   | CXF 243   | 58          | 56           | 73          | 0.3526      | 0.3526      |
Figure 1
Figure 2

A. BI 836880 ▼ ANG2 treated cells ▲ Untreated cells

B. BI 836880 ▼ ANG1 treated cells ▲ Untreated cells

C. BI 836880 ▼ No V\text{HH} ▲ No hAng2

JPET Fast Forward. Published on October 14, 2022 as DOI: 10.1124/jpet.122.001255
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Figure 3

(A) Tumor volume (mm³) over time for different treatments: Vehicle control, Bevacizumab, AMG 386, and BI 836880. The graph shows a linear increase in tumor volume with time for all treatments, with Bevacizumab showing a slight reduction compared to the control.

(B) Tumor volume (mm³) over time for different treatments: Vehicle control, Bevacizumab, AMG 386, and BI 836880. The graph shows a linear increase in tumor volume with time for all treatments, with AMG 386 showing a slight reduction compared to the control.
Figure 4
Figure 5
Figure 6
Supplementary material

Pharmacodynamic and Anti-Tumor Activity of BI 836880, a Dual VEGF and Angiopoietin 2 Inhibitor, Alone and Combined with Programmed Cell Death Protein-1 Inhibition

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**Supplementary Table S1.** Mutational status, histology and origin of patient-derived xenograft models.

| Indication | Model     | Mutation status (Oncocarta panel) | Histology          | Origin                  |
|------------|-----------|-----------------------------------|--------------------|-------------------------|
| Pancreas   | PAXF 546  | KRAS: g12V heterozygous           | Adeno-squamous     | Peritoneum              |
|            | PAXF 736  | KRAS: G12R heterozygous           | Adenocarcinoma     | Intestine wall, recurrent|
|            | PAXF 1872 | KRAS G12V heterozygous            | Adenocarcinoma     | Primary                 |
| Lung       | LXFE 211  | CTNNB1: S37F homozygous           | NSCLC, SCC         | Skin metastasis         |
|            | LXFE 1422 | -                                 | NSCLC, SCC         | Primary                 |
|            | NCI-H1975 | CDKN2A E69                        | NSCLC,             | Primary                 |
|            |           | EGFR T790M, L858R                 |                    |                         |
|            |           | PI3KCA G118D                      | Adenocarcinoma     |                         |
|            |           | TP53 R273H                        |                    |                         |
| Breast     | MAXF 401  | TP53: R248W heterozygous          | Adenocarcinoma, basa-like | Lung metastasis |
|            | MAXF 1322 | -                                 | Adenocarcinoma     | Lung metastasis         |
|            | RXF 631   | -                                 | Hyper-nephroma     | Lung metastasis         |
| Organ     | Code       | Genotype/Feature                        | Tumor Type     | Metastasis Site          |
|-----------|------------|-----------------------------------------|----------------|--------------------------|
| Ovary     | OVXF 1353  | -                                       | Adenocarcinoma | Lymph node metastasis    |
| Colon     | CXF 243    | KRAS: G12D heterozygous                 | Adenocarcinoma | Liver metastasis         |

Abbreviations: CDKN2A, cyclin dependent kinase inhibitor 2A; CTNNB1, catenin beta 1; EGFR, endothelial growth factor receptor; KRAS, Kirsten rat sarcoma virus; NSCLC, non-small cell lung cancer; PI3KCA, phosphatidylinositol 3-kinase catalytic subunit; SCC, squamous cell carcinoma; TP53, tumor protein P53.
### Supplementary Table S2. Summary of BI 836880 binding characteristics. Binding affinities were analyzed using a Biacore T100 surface plasmon resonance system or by ELISA.

| Antigen                | $K_D$ [nM]                     | $EC_{50}$ [nM]     |
|------------------------|-------------------------------|-------------------|
| Human VEGF165          | 1.4 (Biacore)                 | 1.4 (ELISA)       |
| Human VEGF121          |                               | 2.3 (ELISA)       |
| Mouse VEGF164          |                               | No binding (ELISA)|
| Cynomolgus VEGF165*    | 1.4 (Biacore)                 |                   |
| Human VEGF-B           |                               | No binding (ELISA)|
| Human VEGF-C           |                               | No binding (ELISA)|
| Human VEGF-D           |                               | No binding (ELISA)|
| Human PI GF            |                               | No binding (ELISA)|
| Human ANG2-FLD         | 0.016 (Biacore)               |                   |
| Mouse ANG2-FLD         | 0.051 (Biacore)               |                   |
| Cynomolgus ANG2-FLD    | 0.008 (Biacore)               |                   |
| Human serum albumin    | 9 (Biacore)                   |                   |
| Mouse serum albumin    | 150 (Biacore)                 |                   |
| Cynomolgus serum albumin| 9 (Biacore)                  |                   |

$IC_{50}$ [nM]†

| Antigen    | $IC_{50}$ [nM]     |
|------------|-------------------|
| Human ANG1 | >2000             |
| Human ANG2 | 0.05 (with HSA);  |
|            | 0.045 (without HSA)|

*Cynomolgus and human VEGF165 have identical amino acid sequences. †Determined by competition ELISA.

Abbreviations: ANG2, angiopoietin-2; FLD, fibrinogen-like domain; HSA, human serum albumin; PI GF, placental growth factor.
Supplementary Table S3. Least squares means (predicted) and treatment ratio for tumor volume (mm$^3$): results from a repeated measurement mixed model comparing treatment with anti-PD-1 plus anti-VEGFR2 with anti-PD-1 plus anti-VEGFR2 plus anti-ANG2. Female C57BL/6NTac mice bearing LL/2 lung cancer tumors were treated with RMP 1-14 (anti-PD-1 antibody; 10 mg/kg twice-weekly intraperitoneally), vatalanib (anti-VEGFR2 small molecule inhibitor; 100 mg/kg; once-daily by oral gavage), BI 836880 (used as anti-mouse ANG2 nanobody; 15 mg/kg; twice-weekly intraperitoneally), or their combination.

| Day | Treatment                        | LS means (0.95 CI) | Treatment ratio (one-sided 0.95 CI) | $P$ value one-sided |
|-----|----------------------------------|--------------------|------------------------------------|-------------------|
| 1   | Anti-PD1 + anti-VEGFR2           | 57.52 (48.78–67.82)|                                   |                   |
|     | Anti-PD-1 + anti-VEGFR2 + anti-ANG2 | 56.86 (48.22–67.05) | 0.99 (–1.20)                      | 0.4612            |
| 3   | Anti-PD1 + anti-VEGFR2           | 66.47 (56.37–78.37)|                                   |                   |
|     | Anti-PD-1 + anti-VEGFR2 + anti-ANG2 | 66.40 (56.31–78.29) | 1.00 (–1.21)                      | 0.4964            |
| 5   | Anti-PD1 + anti-VEGFR2           | 69.07 (58.57–81.44)|                                   |                   |
|     | Anti-PD-1 + anti-VEGFR2 + anti-ANG2 | 67.55 (57.29–79.65) | 0.98 (–1.19)                      | 0.4252            |
| 8   | Anti-PD1 + anti-VEGFR2           | 71.24 (60.41–84.00)|                                   |                   |
|     | Anti-PD-1 + anti-VEGFR2 + anti-ANG2 | 71.02 (60.23–83.75) | 1.00 (–1.21)                      | 0.4898            |
| 10  | Anti-PD1 + anti-VEGFR2           | 76.58 (64.94–90.30)|                                   |                   |
|     | Anti-PD-1 + anti-VEGFR2 + anti-ANG2 | 76.62 (64.98–90.34) | 1.00 (–1.22)                      | 0.5016            |
|   | Anti-PD1 + anti-VEGFR2 |     | Anti-PD-1 + anti-VEGFR2 + anti-ANG2 |     |     |     |
|---|----------------------|-----|-----------------------------------|-----|-----|-----|
| 12 | 84.20 (71.41–99.29)  |     | 86.05 (72.98–101.47)              | 1.02 (–1.24) | 0.5732 |
| 15 | 98.06 (83.16–115.63) |     | 100.76 (85.45–118.82)             | 1.03 (–1.25) | 0.5913 |
| 17 | 117.59 (99.72–138.65)|     | 120.42 (102.13–142.00)            | 1.02 (–1.24) | 0.5802 |
| 19 | 138.90 (117.80–163.79)|     | 147.77 (125.32–174.25)            | 1.06 (–1.29) | 0.7001 |
| 22 | 187.89 (159.34–221.55)|     | 194.19 (164.68–228.98)            | 1.03 (–1.26) | 0.6102 |
| 24 | 257.54 (218.41–303.68)|     | 229.44 (194.57–270.55)            | 0.89 (–1.08) | 0.1640 |
| 25 | 305.64 (259.20–360.40)|     | 263.92 (223.82–311.21)            | 0.86 (–1.05) | 0.1074 |
| 26 | 357.55 (303.07–421.83)|     | 296.03 (251.05–349.07)            | 0.83 (–1.01) | 0.0559 |

Abbreviations: ANG2, angiopoietin-2; CI, confidence interval; LL/2, Lewis lung carcinoma; LS, least squares; PD-1, programmed cell death protein-1; VEGFR2, vascular endothelial growth factor receptor 2
Supplementary Figure S1.