

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

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Supplementary Data and Methods

Supplementary Data

Supplementary Table S1. mL4-3 selectively neutralizes Ang1:Tie2 interactions

Agent	hAng1 IC₅₀ (nM)	mAng1 IC₅₀ (nM)	rAng1 IC₅₀ (nM)	cAng1 IC₅₀ (nM)	hAng2 IC₅₀ (nM)	mAng2 IC₅₀ (nM)	cAng2 IC₅₀ (nM)
mL4-3	0.045	0.033	0.061	0.039	1876	> 10,000	1890
Fc	> 10,000	> 10,000	> 10,000	> 10,000	> 10,000	> 10,000	> 10,000

h, human; m, mouse; r, rabbit; c, cynomolgus monkey

Supplementary Table S2. Mean pharmacokinetic parameters of angiotensin inhibitors in rodents

Agent	Mouse		Rat	
	$t_{1/2}$ (hr)	Dose-normalized AUC_{0-inf} ($\mu M \cdot hr/mg/kg$)	$t_{1/2}$ (hr)	Dose-normalized AUC_{0-inf} ($\mu M \cdot hr/mg/kg$)
mL4-3	45	5.0	42	3.6
L1-7(N)*	56	7.0	47	4.6
AMG 386*	97	15	85	8.7

*Adapted from Oliner, et al (1).

Supplementary Table S3. Incidence of cardiac abnormalities in mouse embryos treated with Fc or mL4-3

Cardiac Appearance	Interpretation	Incidence n (%)	
		Fc	mL4-3
Normal size, many trabeculae	Normal	9 (56%)	0
Normal size, mildly fewer trabeculae	Normal	6 (38%)	3 (20%)
Normal size, moderately fewer trabeculae	Normal	0	1 (7%)
Reduced size, many fewer trabeculae	Abnormal	1 (6%)	11 (73%)*
	Total	16 (100%)	15 (100%)

* $P < 0.0001$ vs Fc (Chi-square test)

Data were acquired by coded (“blinded”) histopathologic analysis.

Supplementary Table S4. Selective inhibition of Ang1 or Ang2 induces ovarian atrophy but not epiphyseal plate thickening

Agent	Epiphyseal plate thickening (males)	Epiphyseal plate thickening (females)	Ovarian atrophy
AMG 386	10	10	8
L1-7(N)	0	0	8
mL4-3	0	0	6

n = 10 per group; 300 mg/kg twice-weekly IV dosing

Supplementary Figure Legends

Figure S1. Effects of recombinant Ang1, recombinant Ang2, and angiopoietin inhibitors on human umbilical vein endothelial cell (HUVEC) proliferation. **A)** Ang1, **B)** Ang2, and **C)** angiopoietin-inhibiting peptibodies did not alter HUVEC growth rates in IncuCyte live cell imaging assays (Essen).

Figure S2. Effects of recombinant Ang1, recombinant Ang2, and angiopoietin inhibitors on HUVEC permeability. **A)** Ang1, **B)** Ang2, and **C)** angiopoietin-inhibiting peptibodies did not alter the permeability of confluent HUVEC monolayers to horseradish peroxidase (HRP).

Figure S3. Effects of mL4-3 on Tie2 phosphorylation in lung and on vessel phenotypes in heart. **A)** Mice were treated with L1-7(N), mL4-3 or Fc control prior to challenge with Ang1 or BSA. Levels of phosphorylated Tie2 in lungs were determined by immunoprecipitation-Western blot analysis. Data are mean values \pm SE. $*P = 0.0005$ vs Ang1 plus Fc. **B)** Representative images of E12.5 mouse embryos exposed to mL4-3 (right panel) or Fc control (left panel) showing that Ang1 inhibition stunted heart growth and trabecular formation.

Figure S4. The effect of combined Ang1 and Ang2 inhibition on the growth of HT-29 tumor xenografts. HT-29 tumor bearing mice were treated with **A)** Fc control (6 mg/kg QD), L1-7(N) (6.0 mg/kg QD) or AMG 386 (5.6 mg/kg twice per week). Data were not statistically significant. **B)** AMG 386 (5.6 mg/kg twice per week); Fc control (22 mg/kg), L1-7(N) (2.0 mg/kg), or mL4-3 (20 mg/kg) QD; or the combination of L1-7(N) and mL4-3 (at the same dosing regimens used in the single-agent groups). Data are mean values \pm SE. $*P < 0.05$ vs L1-7(N) or mL4-3.

Figure S5. Pericyte association with tumor vessels after inhibition of Ang1 and/or Ang2. **A)** Confocal microscopic images show that pericytes (PDGFR- β , red) associated with endothelial cells of tumor vessels (CD31, green) were sparse after treatment with Fc or mL4-3 for 26 days. L1-7(N) treatment increased pericyte association. The increase in pericytes associated with tumor vessels was not seen after dual inhibition of Ang1 and Ang2 with either the combination of L1-7(N) and mL4-3 or with AMG 386. **B)** A greater abundance of PDGFR- β -positive pericytes within 10 μ m of tumor vessels after L1-7(N) treatment compared to the other groups. * $P < 0.05$ compared with the other groups. Scale bar represents 10 μ m. Data represent mean \pm SE. N= 5 per group.

Supplementary Methods

Pharmacokinetic Assessment

Three CD-1 mice received a single subcutaneous (SC) injection of 3.2 mg/kg of mL4-3, and two Sprague-Dawley rats received a single intravenous (IV) injection of 10 mg/kg of mL4-3. Blood samples for serum pharmacokinetic assessment were collected up to 274 hours (mice) or up to 336 hours (rats). mL4-3 concentrations in serum samples from each species were measured by enzyme-linked immunosorbent assay (ELISA). Polystyrene 96-well plates were coated with human Ang1 (prepared at Amgen Inc., Thousand Oaks, CA), followed by incubation with mL4-3-containing serum samples. After washing away any unbound substances, a horseradish peroxidase (HRP)-labeled monoclonal mouse anti-IgG1 antibody was added to the wells. Following a wash step to remove any unbound monoclonal antibody, tetramethylbenzidine (TMB)-peroxidase substrate was added. The optical density units measured at 450 - 650 nm were converted to concentrations via comparison to a concurrently analyzed standard curve.

Pharmacokinetic parameters were calculated by noncompartmental analysis of the individual serum concentration-time data (WinNonlin Professional, version 3.3; Pharsight Corp, Mountain View, CA). Terminal phase half-life ($t_{1/2}$) was calculated as $t_{1/2} = \ln(2)/\lambda_z$, in which λ_z is the first-order terminal phase elimination rate constant estimated via linear regression of the terminal log-linear decay phase. Area under the serum concentration-time curve (AUC_{0-last}) was estimated by the linear/log trapezoidal method from time 0 to the time of the last quantifiable concentration (C_{last}). AUC_{0-inf} was estimated from time 0 to infinity as $AUC_{0-inf} = AUC_{0-last} + C_{last}/\lambda_z$. AUC_{0-inf} values were normalized to a 1 mg/kg dose.

Angiopietin:Tie2 Neutralization homogeneous time-resolved fluorescence

(HTRF) Assay

Europium-labeled streptavidin (LANCE reagent, PerkinElmer Inc., Boston, MA) and biotinylated human Ang1 (R&D Systems, Inc.) or Ang2 were mixed in HTRF buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.05% Tween 20, 0.1% bovine serum albumin [BSA]) and incubated at room temperature (RT) in the dark for 30 minutes with shaking. Equal volumes of this mixture and serially diluted peptibodies or Fc were mixed and incubated for 1 hour at RT. Equal volumes of allophycocyanin-conjugated Tie2-Fc (Tie2-APC) (Prozyme, San Leandro, CA) and the above mixture were mixed and incubated for 2 hours at RT. The final concentrations of reagents in the assay were 4 nM europium-streptavidin, 2 nM biotinylated Ang1 or Ang2, and 5 nM Tie2-APC. Peptibodies were serially diluted from 10,000 nM to 0.5 nM or 100 nM to 0.005 nM to generate full titration curves. Neutralization of angiopoietin:Tie2 interaction was measured by the diminishing energy transfer between APC and europium and was quantified using a Rubystar plate reader (BMG Labtechnologies, Offenberg, Germany).

Prenatal Ang1 Inhibition

Female 129/SV mice were impregnated by C57BL/6 males. The strains were selected to match those used in genetic Ang1 knockout studies (2). Dams were dosed subcutaneously (SC) with 300 mg/kg Fc control or mL4-3 (n = 6 per group) on embryonic (E) days E4.5, E7.5 and E11.5. Conceptuses (embryos and placentae) were removed on day E12.5, evaluated for gross abnormalities, and fixed by immersion in zinc-tris (3) (mL4-3-treated, n = 10; Fc control-treated, n = 10) or Bouin's solution (mL4-3-treated, n = 5; Fc control-treated, n = 6).

Paraffin-embedded tissues were step-sectioned at 50- μ m intervals through the heart (embryos embedded in both longitudinal and transverse orientation) and the middle of the placenta. Serial sections from each interval were stained with hematoxylin and eosin (H&E) or with a conventional indirect immunohistochemistry procedure using polyclonal anti-CD31 (rat

anti-mouse monoclonal MEC 13.3; BD Biosciences Pharmingen, San Diego, CA) to specifically label blood vessels. Criteria for scoring changes were established by evaluating sections with a foreknowledge of the treatment. Subsequently, lesion severity was graded rapidly by a veterinary pathologist (B.B.) using a tiered scale (minimal, mild, moderate, or marked) and a blinded analytical paradigm. These ordinal pathology data were analyzed using the Chi-square test contained in the JMP statistical software package (v.5.1; SAS Institute Inc., Cary, NC). An embryo from each pregnant mother collected on day E12.5 was analyzed by ELISA using human Ang1 as a capture reagent and HRP-labeled monoclonal mouse anti-IgG1 antibody as a detection reagent. Pharmacokinetic assessment of the mouse embryo lysates demonstrated a mean mL4-3 trough level of 3.0 $\mu\text{g/g}$ of tissue, confirming that mL4-3 was capable of crossing the placenta.

Tie2 Phosphorylation Assay

CD-1 nude mice were treated SC once daily (QD) for 23 days with Fc control (20 mg/kg), mL4-3 (20 mg/kg) or L1-7(N) (2 mg/kg). Mice (n = 3 per group) were then administered 0.48 mg/kg (IV) recombinant human Ang1 (R&D Systems Inc.) or BSA, and Tie2 phosphorylation in mouse lungs was assayed as described (4). Immunoblots were quantified on a Versadoc Imaging System (BioRad, Hercules, CA) using Quantity One software (BioRad) applying local background and linear regression parameters. Phosphorylated Tie2 levels were normalized to total Tie2 levels. Statistical analysis was performed using analysis of variance (ANOVA) followed by Fisher's post hoc test.

Tumor Blood Vessel Area Analysis

Four widely spaced 10 \times objective fields of viable tumor were photographed. Blood vessel and tumor field areas were determined by red/green/blue (RGB) thresholding and automated pixel counting (Visiopharm Integrator System image analysis software; Visiopharm,

Hørsholm, DK). Results were expressed as an area fraction. The same tumor sections were then scanned in their entirety using an Aperio digital slide scanner (Aperio Technologies, Inc., Vista, CA, USA). The images were imported into the Visiopharm Integrator System and the total area of viable tumor in each section determined by RGB thresholding and automated pixel counting.

Retinal Neovascularization

Postnatal (P) day seven (P7) pups and their mothers were placed in a hyperoxic chamber (75% \pm 0.5% oxygen) for 5 days and then returned to room air for an additional 5 days. Chamber temperature was maintained between 20°C and 22°C, and oxygen was constantly controlled by an oxygen control unit (ProOx Model P110 coupled to an oxygen sensor Model E702; Biospherix Ltd, Redfield, NY). One cage with P7 pups remained at room air (normoxia condition). Fc control (200 mg/kg), mL4-3 (100 mg/kg), L1-7(N) (100 mg/kg), or mL4-3/L1-7(N) combination (100 mg/kg each) was administered SC once daily (QD) for nine days starting on P8. From P8 to P11 injections were administered using chamber ports. On P17 the pups were sacrificed; the eyes were removed, fixed using Davidson's fixative, and processed into paraffin using standard methods. Step sections were cut parallel to the optical axis at 100- μ m intervals. Blocks were completely through-sectioned, resulting in 15 or 16 sections per eye. All sections were stained with hematoxylin and eosin (H&E). The middle 10 consecutive slides were used in the analyses, bracketing either side of the optical axis. For each section, the number of vascular nuclei (both endothelial and pericyte nuclei) that were on the vitreous side of the inner limiting membrane were counted. Individual slide counts were recorded and all ten section counts summed for each animal. Five mice in each study group were counted.

Ovarian Follicular Angiogenesis

Four-week old female C57BL/6J mice were injected with 5-7 IU pregnant mare serum (PMS), effectively resetting the estrus cycle. Forty-eight hours later, the mice were injected with 5 IU of human chorionic gonadotropin (HCG) to induce superovulation. The females were then faux-bred to vasectomized males. Fc (300 mg/kg), mL4-3 (150 mg/kg), L1-7(N) (150 mg/kg), or an mL4-3/L1-7(N) combination (150 mg/kg each) was administered SC twice per day for 4 days. Dosing commenced at the time of the initial PMS injection and continued for two consecutive days, with the fourth dose given concurrently with the HCG injection. Mice were euthanized 48 hours following the HCG injection. Right and left ovaries were removed and immersion-fixed in cold zinc tris solution. After 48 hours, ovaries were transferred to 70% ethanol and processed to paraffin using standard methods. Two sequential sections were cut from each ovary pair and individually stained either with H&E or immunostained for vascular endothelium (anti-CD31 antibody MEC 13.3) using DAB as the chromogen. Additionally, the anti-CD31 immunohistochemistry sections were lightly counterstained with hematoxylin. The individual follicles selected for analysis were identified based on transformational state. This was determined by treatment-blind inspection of the H&E sections under low magnification. Corresponding images of ten transformed follicles per animal, where feasible, were then captured at 10x objective magnification from the anti-CD31-immunostained sections using a Nikon FXA microscope equipped with a Nikon DXM1200 digital camera. The follicle section area was delineated as a region of interest, and the CD31-positive area fraction was determined via red/green/blue (RGB) thresholding using MetaMorph image analysis software.

HUVEC Proliferation Assay

HUVECs were grown in EBM-2/EGM-2 growth media (Lonza). Cells from passages 2 to 4 were seeded at 3000 cells/well in 96 well plates and treated with human Ang1 (R&D systems), human Ang2 (Amgen), or peptibodies after cells were fully attached (3 hours post-seeding).

The percentage of confluence was measured by IncuCyte live cell imaging (Essen) at 3- or 4-hour intervals.

HUVEC Permeability Assay

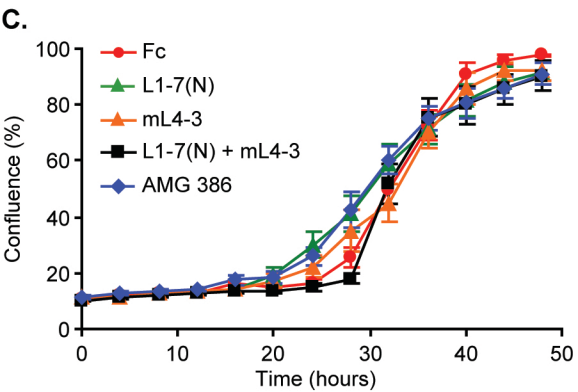
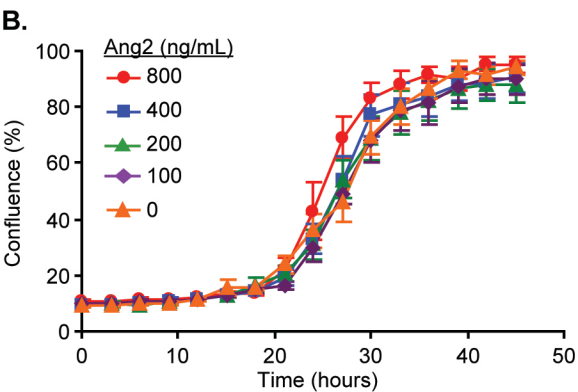
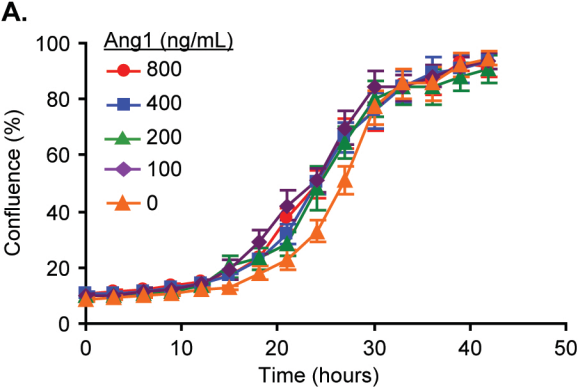
This method was adapted from Rabiet et al (5). HUVECs were grown in EBM-2/EGM-2 growth media (Lonza). Cells from passages 2 to 4 were seeded at 30,000 cells/well on 24-well cell culture inserts (polyethylene terephthalate membrane, 0.4 μ m pores, Becton Dickinson). The inserts were placed into 24-well plates, thereby creating top and bottom media chambers. When cells reached 100% confluence, they were treated with human Ang1 (R&D Systems), human Ang2 (Amgen), or peptibodies in the top chamber for 45 minutes. At the end of treatment, the media in the top chamber was removed, and the inserts were transferred to a new plate containing fresh growth media in the bottom chamber. Growth media containing 0.5 μ M purified horseradish peroxidase (HRP, Thermo Scientific) was added to the top chamber. Aliquots of media from the bottom chambers were taken after 50 minutes of incubation. The activity of HRP in the media was measured by absorbance at 450 nm using TMB substrate (KPL). The amount of HRP in the media was calculated using an HRP standard curve.

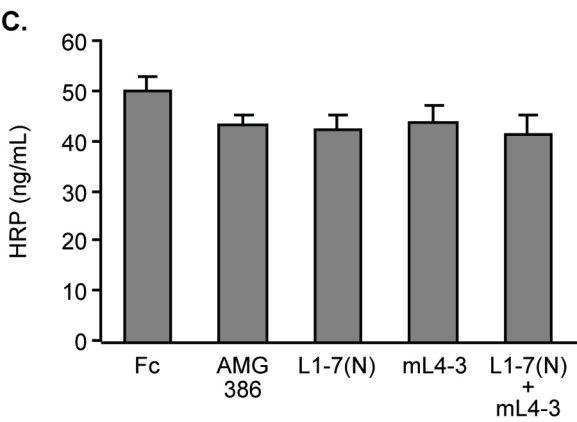
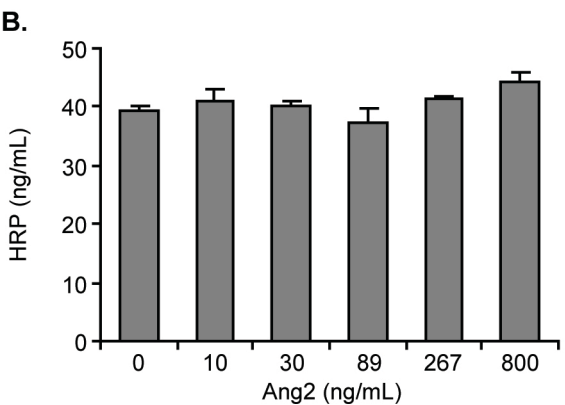
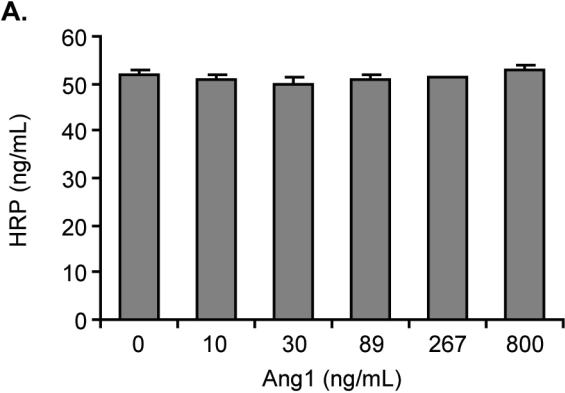
Pericyte distribution on Colo205 tumor vessels

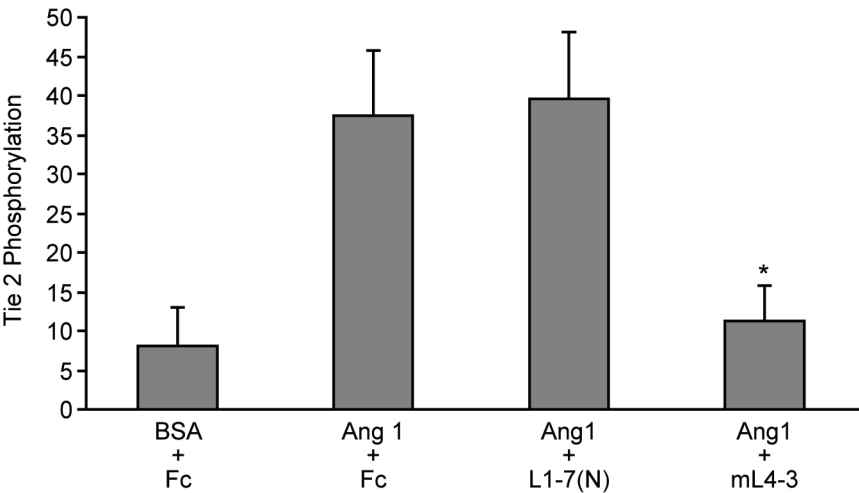
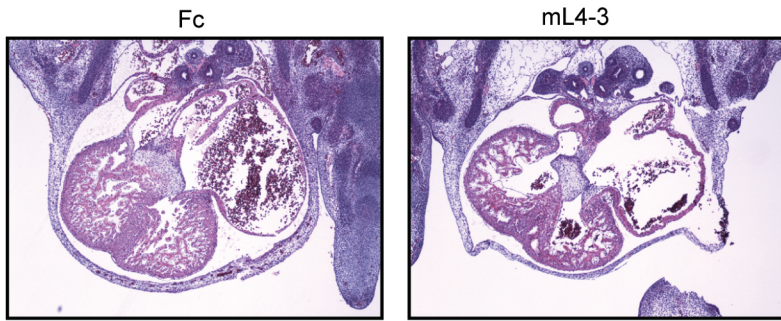
Colo205 tumors were harvested after 26 days of treatment with angiopoietin inhibitors, and immunohistochemistry was performed as described (6). Briefly, tissues were collected from mice perfused with 1% paraformaldehyde and frozen in Tissue-Tek Optimal Cutting Temperature (OCT) compound (Sakura Finetek, Torrance, CA). Cryostat sections (80 μ m) were stained for endothelial cells (anti-CD31; Clone 2H8, 1:500; Thermo Scientific, Hudson, NH) and PDGFR- β (PDGFR- β , clone APB5, 1:2000, eBioscience, San Diego CA) and examined using a Zeiss LSM 510 confocal microscope.

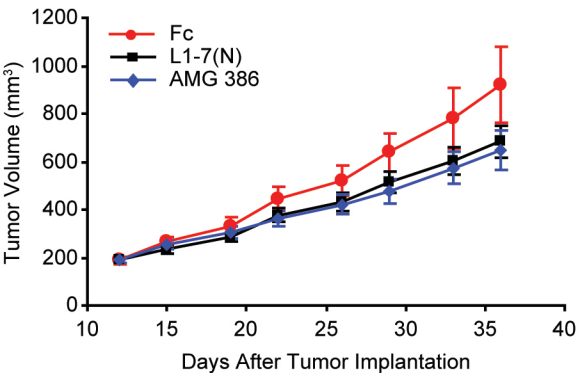
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