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REPORT

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Non-neutralizing antibodies increase endogenous circulating Ang1 levels

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ABSTRACT

Ang1 is a soluble ligand to receptor Tie2, and increasing the circulating Ang1 level may improve vascular stabilization under certain disease conditions. Here, we found that the circulating Ang1 level was significantly increased in cynomolgus monkeys treated with non-neutralizing anti-Ang1 antibodies. Improving the antibodies' pharmacokinetic properties by IgG Fc mutations further increased the circulating Ang1 level. However, the mutations decreased the thermal stability of the molecules, which may limit their use as therapeutic antibodies. Nevertheless, we showed that non-neutralizing antibodies may have therapeutic potential by increasing the level of a target molecule in the circulation.

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Introduction

Monoclonal antibodies (mAbs) are proving to be successful therapeutic agents, owing to their high degree of specificity, favorable pharmacokinetic (PK) and safety profiles, well-established manufacturing processes, and advantageous biophysical properties. Their therapeutic effects against soluble proteins are usually achieved by blocking ligand-receptor interactions with neutralizing antibodies.[1] However, there may be cases in which therapeutic outcomes are achieved by increasing a circulating protein level. Although protein replacement therapy or gene therapy may be considered to boost the circulating level of a soluble protein, there are drawbacks to these procedures, such as poor manufacturability or safety risks.

Neutralizing antibodies often increase the total level of soluble targets. For example, anti-Ang2 neutralizing antibodies increased total Ang2 level up to 30–100 fold relative to baseline level in clinical trials.[2,3] In addition, earlier studies showed that neutralizing antibodies could serve as carrier proteins to increase the target level.[4] When neutralizing antibodies have weaker affinities to soluble ligands than receptors, they may be able to increase the total target level and still allow the receptors to compete for binding to the soluble ligands. Nevertheless, it would be difficult to increase both total level and free level of soluble targets with neutralizing antibodies. In addition, choosing the proper dose for such neutralizing antibodies can be challenging in practice.

Non-neutralizing antibodies may be an attractive alternative if they can increase the level of a soluble protein without affecting the ligand-receptor interactions. Non-neutralizing antibodies can increase the circulating level of a soluble protein by extending its half-life (due to the long half-life of the antibodies) or by sequestering the protein away from its default degradation pathways. Previous animal studies showed that non-neutralizing antibodies extended the half-life of cytokines and enhanced their functional activity, when the antibodies and cytokines were premixed at specific ratios.[5–7] However, cytokines are small, and thus are expected to be removed through renal clearance.[8] Hence, it is possible that the antibodies reduced a cytokine's renal clearance by forming a complex with it, thereby increasing its molecular weight.[9] In any case, it is not clear if non-neutralizing antibodies are effective for increasing soluble proteins that are not cleared by the kidney, or if they can be injected alone (without premixing with the target) to modulate endogenous soluble proteins.

Angiopoietin 1 (Ang1) is a soluble ligand of the receptor tyrosine kinase Tie2, which is primarily expressed on endothelial cells. Ang1 plays important roles in maintaining vascular stability and endothelial barriers by activating the Tie2 receptor, which leads to AKT phosphorylation. Angiopoietin 2 (Ang2), an antagonist to Tie2, competes against Ang1 for Tie2 interaction, inhibiting Tie2 signaling mediated by Ang1.[10] Ang1 contains a fibrinogen-like domain (FLD) that interacts with Tie2's extracellular domain, and a coiled-coil domain (CC) that is involved in Ang1's homodimerization. A cysteine residue between FLD and CC stabilizes the dimer covalently. The Ang1 dimer is further cross-linked in higher-order oligomers by two additional cysteines located in the superclustering motif on the N-terminus (Figure 1). Neither the monomeric FLD domain nor the dimeric CC-FLD proteins are sufficient to activate Tie2 receptors; Ang1's

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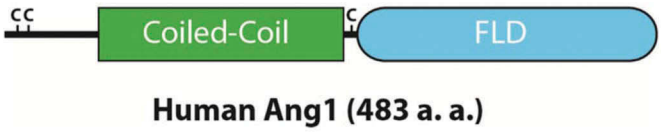

Figure 1. Domain organization of human Ang1: Ang1 contains a coiled-coil domain and a fibrinogen-like domain (FLD). Ang1 forms a homodimer through the coiled-coil domain and interacts with the Tie2 receptor through the FLD domain. On the N-terminus, two cysteine residues (C) cross-link Ang1 to form higher-order oligomers.

function minimally requires tetramers or higher-order oligomers.[11] The Ang1 monomer is 56 kDa, with six predicted N-linked glycosylation sites. Due to the large size of Ang1 oligomers, renal clearance is not expected to be the main clearance pathway for Ang1 in healthy kidneys. Consistent with this expectation, the Ang1 level measured in urine (5 pg/mL) is much lower than that in serum (2500 pg/mL).[12,13]

Ang1 exogenously delivered through an adenovirus vehicle reduced vascular leakage in mice.[14,15] However, the full-length Ang1 protein is difficult to produce and is highly prone to aggregation, making it unsuitable for protein replacement therapy. To overcome production challenges, a stable Ang1 FLD monomer was fused with oligomeric peptides to induce oligomer formation. Such engineered Ang1 fusion proteins have therapeutic effects in mouse models.[16–19] Nevertheless, the Ang1 fusion proteins have disadvantages, including a short half-life, high immunogenic risk, and poor manufacturability, and thus are still challenging to develop as therapeutic agents.[20]

In this study, Ang1 was used as an example to test the concept that the administration of non-neutralizing antibodies alone can increase the circulating level of a soluble protein that does not undergo renal clearance. We demonstrated that non-neutralizing anti-Ang1 antibodies were able to increase the circulating Ang1 level in cynomolgus monkeys.

Results

Non-neutralizing antibody increased the circulating Ang1 level

MAB9231 is a commercially available anti-Ang1 antibody with a mouse IgG2b backbone. MAB9231 binds to human Ang1 CC-FLD (Kd = 1.7 nM) and Ang1 CC (Kd = 1.0 nM), but not to Ang1 FLD (at up to 100 nM). To determine if MAB9231 blocked the Ang1:Tie2 interaction, we developed a blocking assay using Alexa 647-labeled human Ang1 CC-FLD dimer and human embryonic kidney (HEK) cells that overexpressed the human Tie2 receptor. A blocking antibody would prevent Ang1-Alexa 647 from binding to the cells, resulting in a loss of fluorescent signal measured by flow cytometry. MAB9231 did not show blocking activity in this assay (Figure 2a).

To study if MAB9231 could increase the circulating Ang1 level in cynomolgus monkeys, the constant region of the mouse IgG2b (CH1-CH3) was replaced with the corresponding sequence from human IgG1. The binding affinity of the reformatted MAB9231 (MAB9231-RF) for human Ang1 CC-FLD was determined to be 2.4 nM, comparable to that of the original MAB9231, indicating that the reformatted antibody retained its binding ability for Ang1. It should be noted that the mature forms of human Ang1 and cynomolgus Ang1 have identical protein sequences. We next tested MAB9231-RF in a single-dose study at 1.5 mg/kg in cynomolgus monkeys. One male and two female monkeys were treated. The circulating Ang1 levels tended to increase in all three animals, with the male monkey showing the highest Ang1 levels throughout the study (Figure 3). The baseline sample from one animal showed hemolysis, and the baseline Ang1 level from that animal was not measured. For the other two animals, the Ang1 level increased 3.64 fold (Subject 183 Female) and 2.74 fold (Subject 184 Male) over the baseline after 192 hours. Thus, this preliminary study with an engineered commercial antibody provided evidence that non-neutralizing antibodies could increase the circulating Ang1 level. Because the results also indicated a possible gender difference in the overall Ang1 levels, only male monkeys were used in the subsequent experiments.

Generation of therapeutic non-neutralizing antibodies against human Ang1

To develop therapeutic non-neutralizing antibodies that could significantly increase the circulating Ang1 level, transgenic AlivaMab mice were immunized with full-length Ang1 protein. AlivaMab mice produce IgG antibodies with human Fab sequences, while maintaining the mouse Fc to allow a robust immune response.[21] The variable domain sequences from positive B-cell hits against Ang1 were recovered and cloned into a vector that contained a human IgG1 constant region. The molecules were then expressed as human IgG1 antibodies, and their ability to bind Ang1 protein was confirmed. Ang2, which shares 64% sequence identity with Ang1, was included in the selectivity screening. The Ang1:Tie2 blocking assay described earlier was then used to select non-neutralizing antibodies.

As a result of the screening process, an antibody mAB-NN (where NN stands for non-neutralizing) was selected for further characterization. Antibody mAB-NN bound to human Ang1 CC-FLD and human Ang1 CC proteins with Kds of 9.8 nM and 7.3 nM, respectively. Up to 100 nM mAB-NN did not show binding to human Ang1 FLD, suggesting that this antibody binds to the coiled-coil domain. Furthermore, up to 1 μM mAb-NN did not bind to human Ang2 CC-FLD, confirming its specificity for Ang1. In the blocking assay, mAB-NN did not block Ang1's interaction with Tie2 cells (Figure 2a). Notably, MAB9231 enhanced the binding of Ang1 to the Tie2-expressing cells, whereas mAB-NN showed neither inhibition nor enhancement of Ang1:Tie2 interaction. Since Ang1 CC-FLD is a dimer, the bivalent MAB9231 antibody might cross-link Ang1 dimers to higher oligomers, thereby increasing Ang1's binding to the Tie2 cells. Similar clustering antibody has been described for Ang2 that shares similar topology as Ang1.[22] When studied via analytical size exclusion chromatography with a column (e.g., TSKgel G400SWxl) intended to resolve large molecular weight complexes, most MAB9231:Ang1 CC-FLD complex was not recoverable. This result suggests that MAB9231 may cross-link Ang1 dimer to form large aggregates too big to enter the column. In contrast, mAB-NN:Ang1 CC-FLD complex showed a well-defined peak with good recovery (Supplemental Figure 1).

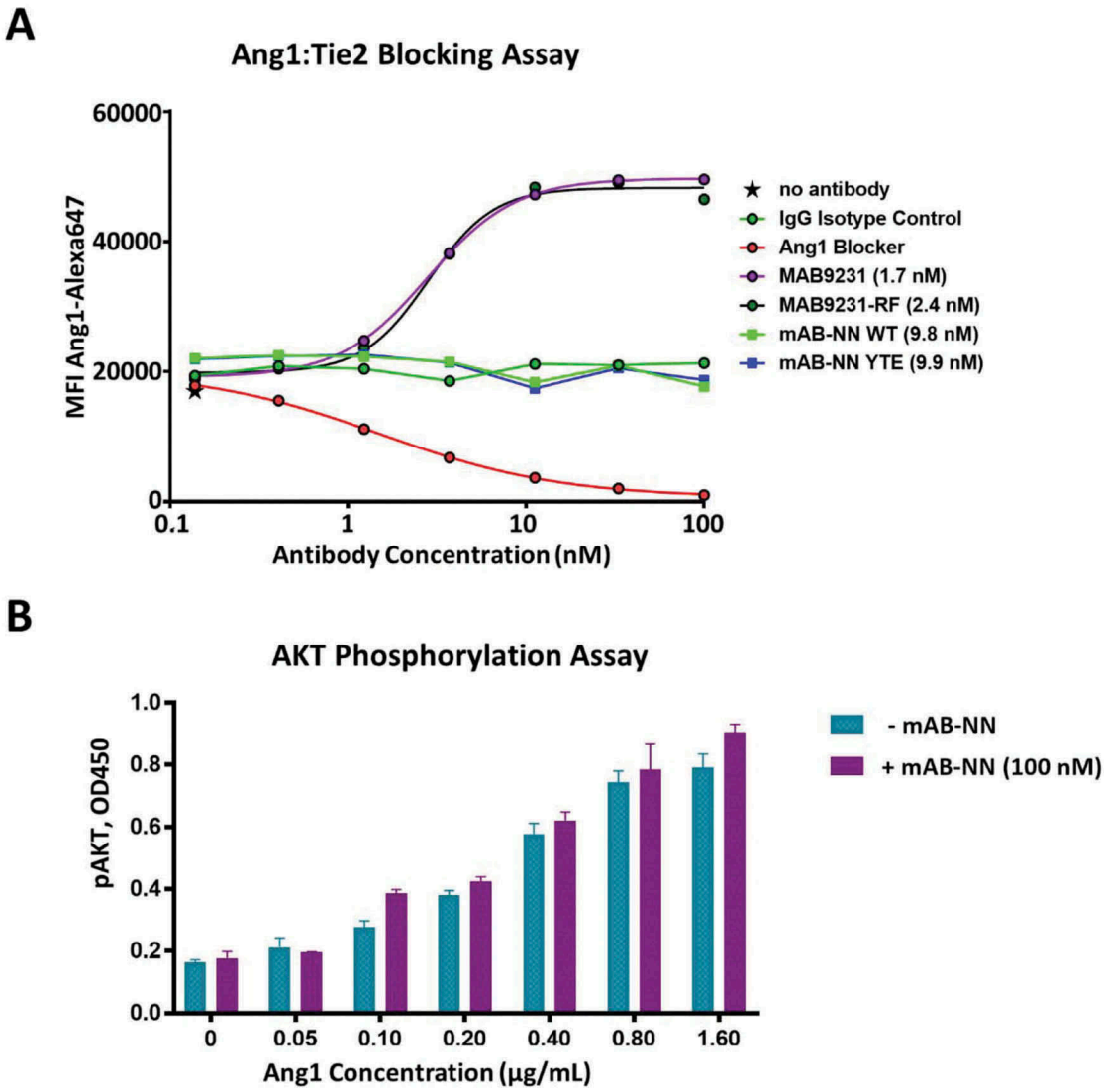

Figure 2. Evaluation of Ang1 non-neutralizing antibodies: (A) Alexa 647-labeled Ang1 CC-FLD dimer was incubated with Tie2-expressing cells in the presence of antibodies: mouse IgG2B MAB9231, reformatted MAB9231 (MAB9231-RF) with human IgG1 Fc, mAB-NN human IgG1 wild type, mAB-NN human IgG1 with YTE mutations, an IgG isotype control, and an Ang1 blocker. MAB9231 and MAB9231-RF enhanced Ang1's binding to the cells. Neither mAB-NN wild type (WT) nor mAB-NN YTE blocked Ang1's binding to the Tie2-expressing cells at concentrations up to 100 nM. (B) AKT phosphorylation resulting from Ang1-mediated Tie2 activation. To mimic the native conditions, full-length human Ang2 was included in the assay at a fixed concentration of 2 µg/mL. Ang2's inhibition of the Tie2 activation and AKT phosphorylation was reversed by increasing the amount of full-length human Ang1. The Ang1-mediated AKT phosphorylation was not affected by the mAB-NN antibody.

Competition binding studies showed that mAB-NN and MAB9231 did not compete for binding to Ang1, suggesting that these antibodies bound to different locations on Ang1 (Supplemental Figure 2). To confirm the data from the blocking assay, mAB-NN was tested in an AKT functional assay with a human endothelial cell line (EA.hy926) that endogenously expressed the Tie2 receptor. Ang2, an antagonist to Ang1, inhibits Ang1-mediated signaling. To mimic the native conditions where both Ang1 and Ang2 are present simultaneously, increasing amounts of recombinant full-length Ang1 were incubated with the cells in the presence of a fixed amount of recombinant full-length Ang2. To test the effects of mAB-NN on Ang1's activity, 100 nM mAB-NN was included in the concentration series. No difference was observed for cells incubated with or without mAB-NN (Figure 2b). Taken together, the binding-assay and functional-assay data demonstrated that mAB-NN does not affect Ang1's binding to the Tie2 receptor, or its activation of downstream signaling.

Cynomolgus monkey PK/PD studies

Next, we studied the effects of the fully human mAB-NN on circulating Ang1 level in cynomolgus monkeys. To test if the PK properties of a non-neutralizing antibody could affect its ability to increase circulating target level, YTE mutations (M252Y, S254T, and T256E) were introduced into mAB-NN.[23] The YTE mutations increase the molecule's binding to FcRn and have been shown to improve antibody PK properties. These mutations are reported to increase the half-life of antibodies in cynomolgus monkey up to 4 fold.[24] We therefore compared mAB-NN in wild-type IgG1 format with mAB-NN in IgG1 with YTE mutations in a multiple-

Circulating Ang1 Levels (pg/mL)

Animal	Pre-dose	0.25 hr	48 hr	120 hr	192 hr
181F	NA*	136.07	163.76	226.99	258.19
183F	74.73	173.14	166.74	241.30	272.27
184M	134.01	273.34	362.73	328.79	367.51
Mean	104.37	194.18	231.08	265.69	299.32
SD	41.92	71.01	114.03	55.11	59.47

*Sample hemolyzed

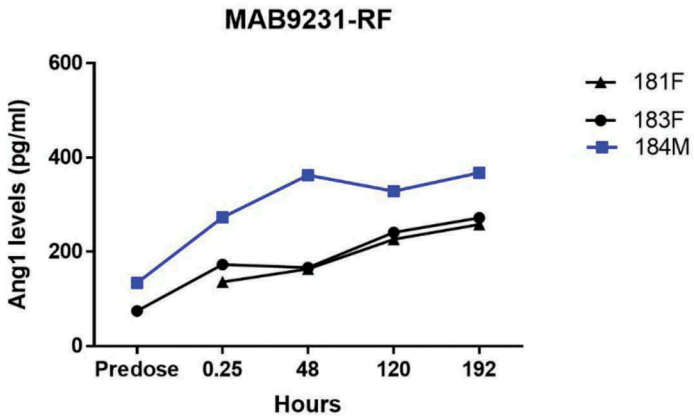

Figure 3. Single-dose cynomolgus monkey study with reformatted MAB9231: Three cynomolgus monkeys were treated with the reformatted MAB9231 antibody at 1.5 mg/kg, and the circulating Ang1 level in each animal was measured over time. The circulating Ang1 level increased in all animals after treatment with the antibody.

dose cynomolgus monkey study. The molecules were administered once a week for three weeks, followed by a post-treatment observation period. Both antibodies elicited increased Ang1 levels through the study period (Figure 4a). Ang1 was increased to a higher level in the animals treated with mAB-NN YTE, probably due to the antibody's higher concentration (Figure 4b). While mAB-NN wild type increased the circulating Ang1 level by 6.7 fold (C_{avg} 0.739 ng/mL vs C_{avg} 0.111 ng/mL for the vehicle group), mAB-NN YTE increased it by 8.7 fold (C_{avg} 0.964 ng/mL vs C_{avg} 0.111 ng/mL for the vehicle group). In contrast, the circulating Ang2 level remained relatively unchanged. (Figure 4c) As a result, the Ang1/Ang2 ratio increased in favor of Ang1 activation.

YTE mutations affect the biophysical properties of the molecule

The YTE mutations improved the PK properties of mAB-NN and boosted the circulating Ang1 to higher level. However, the YTE mutations may affect the biophysical properties of the molecule. To compare mAB-NN wild type and mAB-NN YTE, both antibodies were purified with Protein A. mAB-NN YTE had a lower percentage of monomers than did mAB-NN wild type: 95.58% vs 99.05%, as determined by analytical size exclusion chromatography (Supplemental Figure 3) A second purification step using cation exchange chromatography was then performed to remove aggregates, host cell proteins, DNA, and other contaminants. The YTE mutations are reported to decrease the thermal stability of the molecule.[25] Consistent with this report, the purified mAB-NN YTE had a lower thermal stability than mAB-NN wild type (Figure 5). The onset melting temperature decreased from 57.1°C for mAB-NN wild type to 48.9°C in mAB-NN YTE, an 8-degree decrease. Tm1 (for the CH2 domain) also decreased from 64.9°C to 57.9°C, a 7-degree decrease. In summary, the YTE mutations decreased the thermal stability of mAB-NN.

Discussion

Dysregulation of Ang1/Ang2 ratio has been linked to human disease conditions affected by compromised vascular integrity, including cardiovascular diseases, sepsis, chronic kidney disease (CKD), and systemic lupus erythematosus (SLE).[26–32] In cynomolgus monkey studies, we demonstrated that non-neutralizing antibody mAB-NN wild type increased circulating Ang1 to 6.7 fold and mAB-NN YTE increased Ang1 to 8.7 fold, while keeping Ang2 level relatively unchanged. The level of Ang1 increase is clinically relevant. A previous study showed that increasing severity of chronic heart failure was associated with increase of circulating Ang2 level from 1.627 ng/mL to 6.315 ng/mL (a 3.9-fold increase) and decreasing Ang1/Ang2 ratio from 11 to 2.38 (a 4.6-fold change).[26] Similarly, patients experienced acute

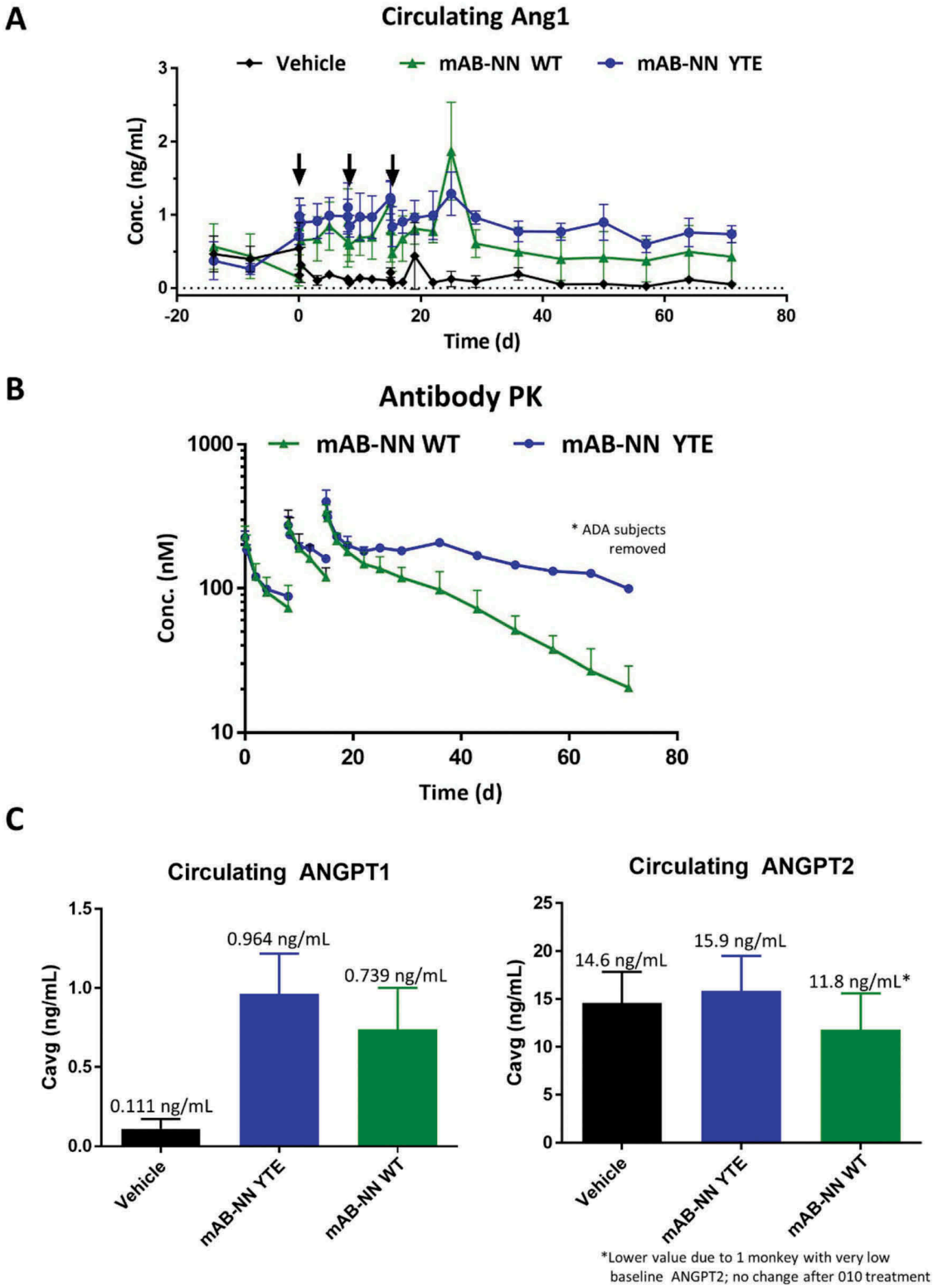

Figure 4. Multiple-dose cynomolgus monkey study with mAB-NN wild type and mAB-NN YTE: Three cynomolgus monkeys were used for each testing group: vehicle, mAB-NN wild type, and mAB-NN YTE. The animals were treated with antibody at 1.5 mg/kg once a week for three weeks (indicated by arrows). The circulating Ang1 level (A) and total antibody level (B) were measured up to 71 days after the first dose. Compared with the Ang1 level, the circulating Ang2 level remained relatively unchanged (C).

myocardial infarction showed baseline Ang2 level averaged to 9.2 ng/mL relative to 3.3 ng/mL in control subjects, while Ang1 was not significantly changed.[27] In a study of CKD patients from different disease stages, Ang2 gradually increased from 0.77 ng/mL in healthy controls to 4.87 ng/mL in CKD 5, and Ang1 did not significantly change.[30] Non-neutralizing antibodies such as

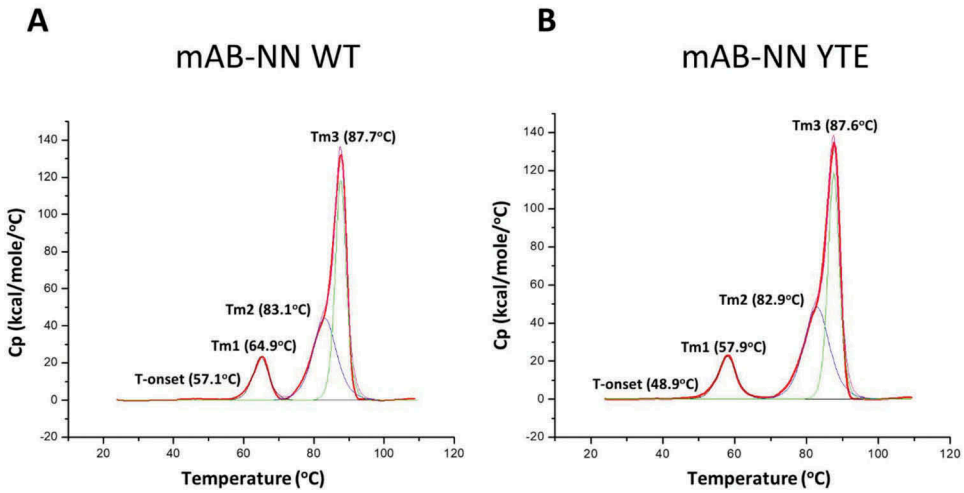

Figure 5. Thermal stability of mAB-NN wild type and mAB-NN YTE: The thermal stability of mAB-NN wild type and mAB-NN YTE was measured by differential scanning calorimetry (DSC). (A) The melting curve for mAB-NN wild type was fitted into three transition phases: Tm1 (64.9oC), Tm2 (83.1oC), and Tm3 (87.7oC). The onset of the thermal melting (T-onset) started at 57.1oC. (B) The melting curve of mAB-NN YTE was fitted into three transition phases: Tm1 (57.9oC), Tm2 (82.9oC), and Tm3 (87.6oC), with 48.9oC for T-onset.

mAB-NN can increase circulating Ang1 and offset the moderate elevation of Ang2, thereby restoring the balance of Ang1/2 ratio to the normal state. In certain conditions such as sepsis and SLE, Ang1 non-neutralizing antibody by itself may not be sufficient to restore Ang1/Ang2 ratio.[28,29,31] However, it may be used in conjugation with Ang2-blocking antibody to further tip the balance of Ang1 and Ang2 in favor of Tie2 activation.

Ang1 exists as oligomers. Bivalent antibodies could potentially cross-link Ang1 to large immune complexes. However, immune complexes most likely form when antibody and antigen have the same molar ratio (e.g., 1 bivalent antibody vs 2 monomeric units of antigen).[32] In the cynomolgus monkey study with mAB-NN, the total Ang1 level stayed below 1.5 ng/mL (except one time point on day 25), which translated to below 27 pM for 56 kD Ang1 protein (Figure 4a). The antibody concentration stayed above 10 nM. Therefore, the antibody was in molar excess (>300 fold) over Ang1 throughout the entire study, an equilibrium condition that did not favor immune complex formation.

By extending the antibody half-life and reducing its clearance, the YTE mutations introduced into mAB-NN increased the circulating Ang1 level beyond what was achieved with the wild-type molecule. This observation suggests that the half-life and clearance of non-neutralizing antibodies influence their ability to increase circulating target levels. However, the YTE mutations can also negatively affect the biophysical properties of the IgGs as previously documented and demonstrated here.[25] A decrease in the IgG monomer percentage after Protein A purification, as we observed for mAB-NN YTE, could decrease the efficiency of subsequent purification steps at the manufacturing scale, leading to lower yields and higher costs. In addition, the decreased thermal stability of the molecule might require more extensive formulation development to find conditions that stabilize the molecule for a long shelf life. Furthermore, since the mutated residues are not naturally present in the constant region of the IgG molecules, they may introduce immunogenicity risks that cannot be fully assessed until a much later stage of clinical development. Therefore, the benefits of using YTE mutations need to be weighed against the risks for each individual target.

In summary, we demonstrated that non-neutralizing antibodies may represent an alternative approach for treating diseases by increasing the circulating level of soluble targets. We showed that, administered alone without premixing with the target, non-neutralizing antibodies could increase the endogenous level of soluble targets. The affinities (1–10 nM) that we tested in the study may not represent the optimal affinities for the concept. The optimal affinities for non-neutralizing antibodies are likely target dependent and influenced by factors such as endogenous target level, target clearance mechanism, target oligomer state and antibody binding epitope/geometry. More studies are needed to test the concept for different targets.

Materials and methods

Reagents

Full-length human Ang1 (Catalog: 923-AN/CF), full-length human Ang2 (Catalog: 623-AN/CF), MAB923 and MAB9231 were purchased from R&D Systems. For the study, MAB9231 was reformatted to contain human IgG1 CH1-CH3 by R&D Systems. Human Ang1 CC-FLD, human Ang1 CC, human Ang1 FLD, and human Ang2 CC-FLD were cloned to contain a C-terminal His_6-tag. The proteins were expressed in HEK cells and purified by Ni-NTA resin and ion exchange chromatography.

Determination of binding affinities

Binding affinities were determined on a ProteOn XPR36 instrument. Antibodies were captured using Protein A/G chips. Human Ang1 CC-FLD, human Ang1 CC, or human Ang1 FLD was titrated at 100 nM, 50 nM, 25 nM, 12.5 nM, and 6.25 nM for 600 sec of association and 1800 sec of dissociation. Human Ang2 CC-FLD was titrated at 1000 nM and 500 nM. Data were fit to a 1:1 Langmuir model.

Ang1:Tie2 blocking assay

Human Ang1 CC-FLD was labeled with Alexa 647. Up to 100 nM antibodies were mixed with 30 nM labeled Ang1 protein. The mixture was incubated for 30 minutes at 4°C before being added to HEK293 cells that express the human Tie2 receptor, for another 30 minutes at 4°C. The cells were washed three times and then subjected to fluorescence signal detection. Antibodies that blocked Ang1's binding to the Tie2 receptor decreased the fluorescence signal.

AKT phosphorylation assay

EA.hy926 cells were starved overnight and then treated for 30 minutes with 2 μg/ml full-length human Ang2 combined with increasing amounts of full-length human Ang1 in the presence or absence of 100 nM mAB-NN. The cells were then lysed and subjected to an ELISA for phospho-AKT.

In vivo pharmacokinetic studies

To study the MAB9231-RF antibody, two female and one male cynomolgus monkeys, naïve to previous treatment with biologics, were administered one intravenous dose at 1.5 mg/kg. Plasma samples were collected before dosing and up to 8 days post-dose. To study the mAB-NN WT and mAB-NN YTE antibodies, male cynomolgus monkeys (n = 3 per dose group), naïve to previous treatment with biologics, were administered once a week for three weeks at 1.5 mg/kg. Plasma samples were collected before dosing and up to 71 days post-dose. The study for MAB9231-RF was conducted in-house, while the studies for mAB-NN WT and mAB-NN YTE were conducted at Charles River Laboratory (Reno, Nevada). Studies were approved by the respective Institutional Animal Care and Use Committees, and were in compliance with US Department of Agriculture Animal Welfare Act (9CFR Parts, 1, 2, and 3).

Determination of total anti-Ang1 mAbs

The plasma concentration of anti-Ang1 antibodies was measured using an ELISA. Briefly, microtiter plates (Nunc) were coated with 1 μg/mL goat anti-Human IgG pre-adsorbed against monkey serum proteins (Southern Biotech, 2049–01). Unbound capture reagent was then washed away (1X phosphate-buffered saline (PBS) with 0.05% Tween 20). The wells were blocked with 5% bovine serum albumin (BSA; Seracare) containing 0.05% Tween 20 and incubated for 1 hour at room temperature. The plates were then washed. Calibration standards, quality control standards, and sample serial dilutions were added, and the mixture was incubated for 1 hour at room temperature. The plates were then washed again. Horseradish peroxidase (HRP)-conjugated goat anti-Human IgG, pre-adsorbed against monkey serum proteins (Novus, NB7489) was added, and the mixture was incubated for 1 hour at room temperature. After washing, the bound HRP-conjugate was detected with a tetramethyl benzidine substrate. The reaction was stopped with 1 M H_2SO_4, and the absorbance was measured using a SpectraMax microplate reader at dual wavelengths of 450 and 650 nm. The signal produced was proportional to the amount of anti-Ang1 antibodies present in the sample. Softmax Pro software (v5.4) was used for calibration standard curve fitting using a 4-parameter logistic model and for the back calculation of all unknown sample concentrations.

Determination of circulating Ang1 level and Ang2 level

The plasma concentration of Ang1 was measured using a Meso Scale Discovery (MSD) electrochemiluminescence assay. Briefly, gold small spot streptavidin plates (MSD part #L45SA) were coated with 1 μg/mL biotinylated mouse monoclonal anti-human Ang1 antibodies (R&D Systems, cat nos MAB923 and MAB9231 biotinylated with EZ-Link Sulfo-LC-Biotin Kit, Thermo Scientific, cat #21,327), then incubated overnight at 2–8°C. For the MAB9231 cynomolgus monkey study, MAB923 was used as the capture reagent, and for the mAB-NN cynomolgus monkey study, MAB9231 was used as the capture reagent. Unbound capture reagent was washed away (1X PBS with 0.05% Tween 20). The plates were blocked with 5% BSA (Seracare) containing 0.05% Tween 20, and incubated for 1 hour at room temperature. The plates were then washed. Calibration standards and sample dilutions prepared in MSD diluent 7 were added, and the mixture was incubated for 2 hours at room temperature. The plates were then washed. MSD Sulfo-labeled polyclonal goat anti-human Ang1 (R&D Systems, AF923) diluted to 1 μg/mL with MSD diluent 3 was added, and the mixture was incubated for 2 hours at room temperature. After washing, 2x MSD Read Buffer was added, and the electrochemiluminescence signal was then measured on a Sector Imager 6000 instrument. Unknown plasma concentrations were calculated from standard curves fitted to a four-parameter logistics equation using the MSD Discovery Workbench software.

The plasma concentration of Ang2 was determined with commercial MSD kit (R-PLEX Human Ang-2 Antibody Set, Catalog: F21YR-3).

Thermal stability

Melting temperatures of the purified antibodies (in 60 mM acetate, 150 mM NaCl, pH 5.0) were determined by differential scanning calorimetry. The temperature was increased at 1°C/sec. Data were analyzed with Origin software.

Analytical size exclusion chromatography

The monomer percentage of the antibodies were measured on Acquity UPLC BEH200 column (200Å, 1.7 μm, 4.6 × 150 mm). The data was analyzed with Empower software. To study the complex formation of antibodies and Ang1, either MAB9231 or mAB-NN was mixed with Ang1 CC-FLD at 1:2 molar ratio. The complex and equivalent amount of antibody alone or Ang1 alone samples were tested on TSKgel G400SWxl column.

Abbreviations

Ang1	Angiopoietin 1
CC	Coiled-coil Domain

FLD	Fibronectin-like Domain
mABs	Monoclonal Antibodies
NN	Non-neutralizing
PK	Pharmacokinetic
Tie2	Tyrosine-protein Kinase Receptor 2

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Disclosure of Potential Conflicts of Interest

The authors declare that they have no conflicts of interest with the contents of this article. At the time when this research work was conducted, all authors were employees of Boehringer Ingelheim Pharmaceuticals Inc.

Notes on contributor

CZ is the Ang1 project leader for the antibody drug discovery and lead author for the manuscript, JT provided PK modeling, TB performed the PK bioassay, MM performed the pAKT functional assay, KJ performed the SPR binding and thermal stability study, KB performed the Tie2 blocking assay, JS coordinated in house cyno study for MAB9231, PS performed the in vivo mouse study to support the project, data not included, DH performed the analytical ultracentrifugation study (AUC) to characterize immune complex, data not included, RF is the senior biology lead for the project, SS and RKB are originators of the concept of using non-neutralizing antibody to increase Ang1.

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