Rapid Development of Glaucoma Via ITV Nonselective ANGPT 1/2 Antibody: A Potential Role for ANGPT/TIE2 Signaling in Primate Aqueous Humor Outflow

Evan A. Thackaberry,1,2 Yi Zhou,1 Christina L. Zuch de Zafra,1,3 Germaine Fuh,1,4 Chingwei V. Lee,1,4 Sarah Sanowar,1,5 John B. Ridgway,1 Aija M. Kusi,1 Cindy Farman,1,6 Helen Booler,1 Daniel Sheinson,1 Carol A. Rasmussen,7,8 Paul E. Miller,7,9 Eric Wakshull,1 Minhong Yan,1 and Vladimir Bantseev1

1Genentech Inc., South San Francisco, California, United States
2Ra Pharmaceuticals, Cambridge, Massachusetts, United States
3Amgen, South San Francisco, California, United States
423andMe, South San Francisco, California, United States
5Verily Life Sciences, South San Francisco, California, United States
6Farman Pathology, Reno, Nevada, United States
7Ocular Services on Demand, LLC (OSOD), Madison, Wisconsin, United States
8Department of Ophthalmology and Visual Sciences, School of Medicine and Public Health, University of Wisconsin-Madison, Madison, Wisconsin, United States
9Department of Surgical Services, School of Veterinary Medicine, University of Wisconsin-Madison, Madison, Wisconsin, United States

Correspondence: Vladimir Bantseev, Department of Safety Assessment, Genentech, Inc., 1 DNA Way, South San Francisco, CA 94080, USA; bantseev@gene.com.

EAT and YZ contributed equally to the work presented here and should therefore be regarded as equivalent authors.

Submitted: December 6, 2018
Accepted: August 1, 2019

PURPOSE. Investigate a significant, dose-related increase in IOP, leading to glaucomatous damage to the neuroretina and optic nerve following intravitreal (ITV) administration of a bispecific F(ab’)2 [anti-VEGF/Angiopoietins [ANGPT]F(ab’)2] molecule in adult monkeys.

METHODS. ITV ocular tolerability and investigation of anti-VEGF/ANGPT F(ab’)2 (blocking both ANGPT1 and ANGPT2) was done in monkeys; mechanistic studies were done in neonatal mice.

RESULTS. Following the second ITV dose of anti-VEGF/ANGPT F(ab’)2, all 1.5- and 4-mg/eye treated monkeys developed elevated IOP, which eventually was associated with optic disc cupping and thinning of the neuroretinal rim. Histopathologic examination showed nonreversible axonal degeneration in the optic nerves of animals administered 1.5 mg/eye and higher that was considered secondary to high IOP. Anti-ANGPT Fab also caused elevated IOP in monkeys, but anti-VEGF Fab did not contribute to the IOP increase. In addition, an anti-ANGPT2-selective antibody did not change IOP. In mice simultaneous blockade of ANGPT1 and ANGPT2 impaired the expansion and formation of Schlemm’s canal (SC) vessels, similar to genetic ablation of Angpt1/Angpt2 and their receptor TIE2. As previously reported, blocking ANGPT2 alone did not affect SC formation in mice.

CONCLUSIONS. Dual inhibition of ANGPT1/ANGPT2, but not ANGPT2 alone, leads to increased IOP and glaucomatous damage in monkeys. This confirms a role for TIE2/ANGPT signaling in the control of IOP in adults, a finding initially identified in transgenic mice. Dual pharmacologic inhibition of ANGPT1/ANGPT2 may affect aqueous drainage and homeostasis in adult monkeys and may be useful in developing novel models of glaucoma.

Keywords: drug development, intravitreal injection, intraocular pressure, aqueous humor outflow homeostasis, ocular toxicology, ocular pharmacology

Angiopoietins (ANGPTs) are a family of secreted glycoproteins known to be involved in embryonic and postnatal angiogenesis and vascular homeostasis. Genetic deletion of angiopoietin 1 (Angpt1) or its receptor Tie2 (a ubiquitous endothelial receptor tyrosine kinase) is embryonic lethal, resulting in severe vascular malformations. Initial studies identified ANGPT1 as a potent TIE2 agonist, while ANGPT2 can act either as a weak agonist or an antagonist to ANGPT1 signaling, depending on cellular context. Many groups have shown that increased ANGPT1 signaling via TIE2 leads to increased vascular stability and reduced permeability. Interestingly, genetic deletion of ANGPT2 is not embryo-lethal, but does result in defective vascular remodeling and significant lymphatic defects. The vascular remodeling defects cannot be rescued with the addition of Angpt1, but Angpt1 is able to rescue the lymphatic phenotype. This suggests that ANGPT/TIE2 signaling is fundamentally different in lymphatic vessels, with both ANGPT1 and ANGPT2 acting as TIE2 agonists.
Further evidence for this context-specific biology was provided by Thomson et al., who demonstrated that simultaneous inactivation of both Angpt1 and Angpt2 during specific periods of mouse embryonic development (E16.5) leads to ocular hypertension and glaucoma due to compromised drainage from the eye due to failure of Schlemm’s canal (SC) to develop and loss of lymphatic capillaries in the corneal limbus. Subsequent work demonstrated that dual inhibition of ANGPT1/2 signaling could also induce ocular hypertension in adult mice and that this was also associated with impaired maintenance of SC integrity. In a more recent study, Thomson et al. demonstrated that knockout mice in which both Angpt1 and Angpt2 was missing had complete absence of SC and a marked glaucomatous phenotype, whereas in those missing only Angpt1 SC development was limited and the glaucomatous phenotype was less severe. Mice, in which only Angpt2 had been knocked out, had no apparent abnormality in SC development or IOP. This suggests that Angpt1, but not Angpt2 is required for the development of SC in mice.

Several groups have reported the involvement of ANGPT/TIE2 signaling in adult vascular diseases, including AMD and diabetic macular edema (DME). This involvement presents an intriguing therapeutic target, potentially synergistic with the current anti-VEGF therapies. This has led to the investigation of selective ANGPT2 and dual ANGPT1/ANGPT2 antagonists as potential antiangiogenic therapeutics for neovascular diseases of the eye.

In order to target both VEGF and ANGPT as a potential treatment for AMD, our group developed a bispecific F(ab)’2 (VEGF/ANGPT F(ab)’2) molecule. During nonclinical safety testing of our intravitreally (IVT) administered VEGF/ANGPT Fab’2 in cynomolgus monkeys, no adverse systemic effects were observed. However, a significant dose-related increase in IOP was observed, leading to glaucomatous damage to the optic nerve and neuroretina. Follow-up studies suggest that the increase in IOP is due to simultaneous blockade of ANGPT1 and ANGPT2 signaling and that selectivity for ANGPT2 over ANGPT1 is a critical safety factor in the design of therapeutics targeting this pathway in the eye.

This observation is consistent with previous work in adult transgenic mice in which inhibition of Angpt-Tie2 signaling was associated with altered integrity of SC and glaucomatous damage. Therefore the Angpt-Tie2 system is not only important for the normal development of SC but also is critical for the maintenance of its integrity in adult mice and cynomolgus monkeys. The effects of these factors on IOP may be related to the fact that, although SC originates from the choroidal vein, it postnatally acquires a number of morphologic, functional, and molecular similarities with lymphatic vessels, and hence is a mixture of vascular and lymphatic identities.

**Materials and Methods**

**Antibody Production**

Generation of anti-VEGF Fab, anti-ANGPT Fab, anti-VEGF/ANGPT bispecific F(ab)’2, and anti-ANGPT IgG have been described previously. Nucleic acid sequences were cloned into Escherichia coli expression vectors and expressed. For Fab generation, human IgG1 half-antibodies (anti-VEGF or anti-ANGPT) were digested 1/1000 (wt/wt) with the endopeptidase Lys-C (Wako Chemicals, Richmond, VA, USA) at 37°C and pH 7.5 for 3 hours or until the Fab had been completely cleaved. The protease was then deactivated by the addition of a 100 M excess inhibitor-to-enzyme ratio with N-tosyl-L-lysine chloromethyl ketone hydrochloride. The digest products were subsequently purified by cation exchange using SP HP (GE Healthcare, Marlborough, MA, USA) with a 30-column volume (CV) gradient from 0 to 300 mM NaCl in 50 mM sodium acetate in pH 5.0. Bispecific human anti-VEGF/anti-ANGPT F(ab)’2 was generated by digesting bispecific antibody assembled from half antibodies. Pepsin was added at 1/500 at pH 3.0 for 2 hours or until the Fab had been removed. The pH of the material was then adjusted to 5.0, and the digests were purified by cation exchange using SP HP (GE Healthcare) and a 30-CV gradient from 0 to 300 mM NaCl in 50 mM sodium acetate at pH 5. The material was subsequently purified by S75 Superdex Gel Filtration (GE Healthcare) to formulate and remove any undigested antibody or fragments. To produce ANGPT2 specific antibody (anti-ANGPT2), the variable domains of anti-ANGPT2 antibody were cloned into mammalian expression vectors with human light chain or heavy chain constant domain for transient IgG expression in Chinese hamster ovary (CHO) cells. An ANGPT1/ANGPT2 cross-reactive antibody (anti-ANGPT) was similarly produced. Full-length IgG proteins were purified using protein A, followed by ion exchange chromatography, and quantified by spectrophotometry at 280 nm.

**Biochemical Characterization**

**Affinity Determinations.** For affinity determinations, the Fab was used as the analyte in BIACore SPR measurements using a CM5 sensor chip immobilized with low density (RU) of human VEGF8-109 (hVEGF109), the receptor binding domain of human ANGPT2 with a C-terminal his-tag (hANGPT2his) or human Fc C-terminal fused to the receptor binding domain of human Ang1 protein (Fc.ANGPT1) at 25°C to determine monovalent affinities. Association rates (k on) and dissociation rates (k off) were calculated using a one-to-one Langmuir binding model and equilibrium dissociation constant (K D) as the ratio of k on/k off.

**HUVEC Assay and Immunoblotting.** Low pass human umbilical vascular endothelial cells (HUVEC) were grown in 6-well tissue culture plates to confluence and then incubated in basal medium EBM-2 (Lonza, Rockland, ME, USA) with 0.1% bovine serum albumin (BSA; Sigma-Aldrich Corp., St. Louis, MO, USA) overnight. Cells were stimulated with VEGF (Genentech, Inc., South San Francisco, CA, USA), ANGPT2 (R&D Systems, Minneapolis, MN, USA), or ANGPT1 (R&D System) with or without antibodies at various dose levels and then washed once in ice-cold PBS. Cells were lysed with RIPA lysis buffer (1M Tris pH 7.4, 4M NaCl, 0.25M EDTA, 0.1% SDS and 1% NP40). An anti-phosphorylated-VEGFR2 or total VEGFR2 (#2471 and #2472, respectively; Cell Signaling Technology, Danvers, MA, USA), phosphorylated-TIE2 or total TIE2 (#4221 and #4224; Cell Signaling Technology), and phosphorylated-ACK or total AKT (#9271S and #9278S; Cell Signaling Technology) were used for immunoblotting.

HUVEC migration assays were performed using Falcon 24-multiswell insert systems (BD Biosciences, Bedford, MA, USA). The inserts were precoated with mouse laminin (Life Technologies, Carlsbad, CA, USA) overnight. HUVECs were starved overnight, harvested, and resuspended in assay medium (EBM-2, 0.1% SA). Cells (5 × 104) were added to the upper chamber, and 20 ng/ml of VEGF was added to the lower chamber to stimulate migration in the presence or absence of various dose levels of blocking antibodies for 16 hours. After fixing and scraping from the upper face membrane, cells on the lower face were fixed with methanol and stained with Sytox green (Life Technologies). Images were acquired using an inverted fluorescent microscope, and cell number was analyzed using ImageJ software (http://imagej.nih.gov/ij/).
provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

**Measuring pAkt in Rat Aortic Endothelial Cells (RAEC).** RAECs (Vect Technologies, Inc., Rensselaer, NY, USA) were cultured in EGM2 MV culture media (Lonza). Cells were seeded at 13,000 cells/well in Costar 96-well, flat-bottom tissue culture plates (Corning, Inc., Life Sciences, Tewksbury, MA, USA) the day before the assay. The next day, cells were incubated in EGM2 MV basal media (EBM2 with 0.2% BSA) for 3 to 4 hours. Test reagents were diluted into the same media and added to cells in triplicate. After 15-minutes incubation, the media was removed and cells were lysed in 40 μL of lysis buffer (Cisbio HTRF pAkt [Ser473] kit, Cisbio U.S., Inc., Bedford, MA, USA). pAkt level was measured according to manufacturer’s instructions.

**In Vivo Evaluations.**

All procedures conducted in animals complied with the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the Office of Laboratory Animal Welfare. Protocols were approved by the local Institutional Animal Care and Use Committee (Covance Laboratories Inc. for NHP studies; Genentech Inc. for mouse studies) and done in accordance with the ARVO Statement for the Use of Animals in Ophthalmology and Vision Research.

**Cynomolgus Monkey Studies.** Naïve male and female cynomolgus monkeys (Covance Research Products, Inc., Madison, WI, USA) were assigned to the pivotal and follow-up investigational studies. The main study was performed in accordance with U.S. Food and Drug Administration Good Laboratory Practice (GLP) regulations. Animals were 30- to 41-months old and ranged in weight from 2.3 to 3.4 kg at study entry. Following anesthesia, and following aseptic preparation of the ocular surface, repeat-dose bilateral ITV injections administered as a 50-μl bolus/eye of sterile saline, 2-mg/eye anti-VEGF Fab, 2-mg/eye anti-ANGPT Fab, or 3-mg/eye anti-ANGPT2 mAb treatments every 2 weeks (Q2W) on days 15, 29, 43, 57, and 71. On Day 72, three animals/sex/group were necropsied (terminal necropsy) and the remaining two animals/sex/group were followed for an additional 4-week recovery (recovery necropsy).

**Follow-Up Investigational Cynomolgus Monkey Study.** Female monkeys (2 control animals; 3/treatment group) under general anesthesia and following aseptic preparation of the ocular surface received repeat-dose bilateral ITV injections administered as a 50-μl bolus/eye of sterile saline, 2-mg/eye anti-VEGF Fab, 2-mg/eye anti-ANGPT Fab, or 3-mg/eye anti-ANGPT2 mAb treatments every 2 weeks (Q2W) on days 1, 15, and 29.

Assessment of toxicity was based on mortality, clinical observations, qualitative food consumption, body weights, ophthalmic examinations (using a slit-lamp biomicroscope and indirect ophthalmoscope), IOP measurements, anterior-segment (AS) OCT, aqueous humor formation (AHF) assessment, and clinical and anatomic pathology. Serum samples were collected for toxicokinetic and antidrug antibody evaluations. A selected set of tissues was collected and processed for H&E staining and subsequent microscopic analysis. Ocular tissues were embedded and sectioned to facilitate examination of the injection site, optic disc, and optic nerve. Sectioning of the eye included a cross section of the optic nerve in addition to the standard section. Sections were analyzed by a board-certified veterinary anatomic pathologist.

**Aqueous Humor Formation.** AHF assessment was conducted once during the predose phase and during study weeks 3 and 5. On the day prior to testing, animals were anesthetized with ketamine once during the predose phase and during weeks 3 and 5. The size of the iridocorneal angle was measured by placing a maximum phantom circle on an image taken of the iridocorneal angle with the point of contact just behind Schwalbe’s line and estimating the largest diameter circle that was accommodated.

**Main GLP Cynomolgus Monkey Study.** Male and female monkeys (5/sex/group) received repeat-dose bilateral ITV injections of vehicle or anti-VEGF/ANGPT F(ab’)2 at dose levels of 0.5, 1.5, or 4 mg/eye/dose every 2 weeks on Days 1, 15, 29, 43, 57, and 71. On Day 72, three animals/sex/group were necropsied (terminal necropsy) and the remaining two animals/sex/group were followed for an additional 4-week recovery (recovery necropsy).

The assessment of toxicity was based on mortality, clinical observations, qualitative food consumption, body weights, ophthalmic examinations (using a slit-lamp biomicroscope and indirect ophthalmoscope), IOP measurements, full-field electroretinograms (ERGs), optical coherence tomography (OCT), fluorescein angiography (FA), fundus ocular photography (OP), and clinical and anatomic pathology. Serum samples were collected for toxicokinetic and antidrug antibody evaluations. At necropsy, an examination of the external features of the carcase, external body orifices, abdominal, thoracic, and cranial cavities, organs, and tissues was performed. A selected set of tissues was collected and processed for hematoxylin and cosin (H&E) staining and subsequent microscopic analysis. Ocular tissues were embedded and sectioned to facilitate examination of the injection site, optic disc, and optic nerve. Sectioning of the eye included a cross section of the optic nerve in addition to the standard section. Sections were analyzed by a board-certified veterinary anatomic pathologist.

Enhanced high-resolution cornea, enhanced AS single- and high-resolution cornea scans were collected with the Visante time-domain OCT (AS-OCT) instrument (Carl Zeiss Meditec, Inc., Dublin, CA, USA) conducted on animals anesthetized with ketamine once during the predose phase and during weeks 3 and 5. The size of the iridocorneal angle was measured by placing a maximum phantom circle on an image taken of the iridocorneal angle with the point of contact just behind Schwalbe’s line and estimating the largest diameter circle that was accommodated.

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TABLE 1. List of All Antibodies Used With Cross-reference to Specific Figures

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Target</th>
<th>ANGPT1</th>
<th>ANGPT2</th>
<th>VEGF</th>
<th>Figures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-VEGF/ANGPT F(ab')2</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1-5</td>
</tr>
<tr>
<td>Anti-ANGPT</td>
<td></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>7</td>
</tr>
<tr>
<td>Anti-ANGPT Fab</td>
<td></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>2, 6</td>
</tr>
<tr>
<td>Anti-ANGPT2</td>
<td></td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>1, 2, 7</td>
</tr>
<tr>
<td>Anti-VEGF Fab</td>
<td></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>2</td>
</tr>
</tbody>
</table>

**Mechanistic Mouse Studies**

CD-1 mice were purchased from Charles River Laboratories (Wilmington, MA, USA). Neonatal mice from the same litter were intraperitoneally dosed with an ANGPT1/ANGPT2 cross-reactive antibody (anti-ANGPT) or an ANGPT2 specific antibody (anti-ANGPT2) at 10 mg/kg on postnatal day (P) 1, P3, and P5. Anti-GP120 was used as a control antibody. Necropsies were performed on P7.

**Anterior Segment Flat Mount.** Eyes were enucleated and fixed in 4% paraformaldehyde (PFA) in PBS overnight at 4°C. After fixation, eyes were washed three times in PBS to remove residual PFA, and the eyes were further dissected. An annular incision was made around the limbus region, and the ciliary body, iris, and lens were carefully removed. The anterior eyecup containing the SC and the cornea was cut radially to flat mount onto slides.25

**Immunofluorescence and Microscopy.** The anterior eyecups were permeabilized with 0.5% Triton X-100 in PBS (PBST) for 15 minutes and washed three times with PBS. Samples were then blocked with 10% normal goat serum in PBST for 1 hour at room temperature, and incubated with primary antibodies diluted in antibody diluent solution (Invitrogen, Carlsbad, CA, USA) overnight at 4°C for with gentle rocking. Following primary antibody incubation, samples were extensively washed in PBST for more than 6 hours, and incubated with Alexa Fluorophore-conjugated secondary antibodies (Invitrogen) at 4°C for overnight with gentle rocking. After extensive washing in PBST, samples were then counterstained with 4',6-diamidino-2-phenylindole (Molecular Probes, Eugene, OR, USA) and flat-mounted onto slides in VECTASHIELD mounting medium (Vector Laboratories, Burlingame, CA, USA). The following primary antibodies were used: rat-anti-Endomucin (clone V7C7.1; Abcam, Cambridge, MA, USA) and rabbit-anti-Prox1 (clone 70R-PR039; Fitzgerald Industries International, Acton, MA, USA). Images were obtained with a Leica SP5 laser scanning confocal microscope (Buffalo Grove, IL, USA).

**Image Processing and Statistics.** Confocal microscope scanned images stacks were processed with Imaris software (Bitplane, Concord, MA, USA). To quantify the area of SC, a surface rendering with local contrast background subtraction was performed with Endomucin staining. The area of Endomucin positive staining in an image grid of 200 × 388 × 21 µm was measured as the area of SC per image grid. Group differences were calculated with an unpaired two-tailed Student’s t-test. P < 0.05 was considered as statistically significant.

**RESULTS**

Table 1 lists all antibodies used in this work, with cross-references to specific figures. The binding characteristics of the antibodies tested in vivo are described in Tables 2 and 3, with ranibizumab (anti-VEGF Fab) included as a control. The inhibitory activity (IC50) of the anti-VEGF/ANGPT F(ab')2 in the VEGF-induced HUVEC cell migration assay was 0.37 nM, which is similar to ranibizumab (0.41 nM, Table 2). While the anti-VEGF/ANGPT F(ab')2 and anti-VEGF Fab bound to VEGF with similar affinity to ranibizumab, the anti-ANGPT Fab or a different anti-ANGPT monoclonal antibody (anti-ANGPT2) did not bind (Table 2). Similarly, the anti-VEGF/ANGPT F(ab')2
TABLE 3. Characterization of ANGPT1 and ANGPT2 Affinities for the Antibodies

<table>
<thead>
<tr>
<th>Molecule</th>
<th>ANGPT2 $K_{on}$ (10$^6$ M$^{-1}$s$^{-1}$)</th>
<th>ANGPT2 $K_{off}$ (10$^{-9}$ M$^{-1}$s$^{-1}$)</th>
<th>ANGPT2 $KD$ (nM)</th>
<th>ANGPT1 $KD$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-VEGF Fab</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Anti-VEGF/ANGPT F(ab')2</td>
<td>1.68 ± 0.28</td>
<td>2.84 ± 0.01</td>
<td>0.18 ± 0.07</td>
<td>115 ± 5</td>
</tr>
<tr>
<td>Anti-ANGPT Fab</td>
<td>2.68 ± 0.18</td>
<td>2.74 ± 0.01</td>
<td>0.1 ± 0.25</td>
<td>107 ± 8</td>
</tr>
<tr>
<td>Anti-ANGPT2</td>
<td>0.32 ± 0.1</td>
<td>0.57 ± 0.01</td>
<td>0.17 ± 0.02</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, no binding detected.

and the anti-ANGPT Fab bound to ANGPT2 with similar affinities, while no ANGPT2 binding was detected with the anti-VEGF Fab (Table 3). Both the anti-VEGF/ANGPT (Fab')2 and the anti-ANGPT Fab also bound to ANGPT1, although this interaction was approximately 600- to 1000-fold weaker than the interaction with ANGPT2. A different anti-ANGPT monoclonal antibody (anti-ANGPT2) showed similar affinity against ANGPT2 as the anti-VEGF/ANGPT F(ab')2 and anti-ANGPT Fab, but no detectable binding to ANGPT1 or VEGF (Table 3). The ANGPT1 cross-reactivity of anti-VEGF/ANGPT F(ab')2 was confirmed in a cell-based assay. ANGPT1 induced strong phosphorylation of AKT (pAKT) in primary RECs. The ANGPT2 specific antibody, anti-ANGPT2, had little effect on the ANGPT1-induced pAKT. In comparison, anti-VEGF/ANGPT F(ab')2 inhibited the ANGPT1-induced pAKT in a dose-dependent manner (Fig. 1). Therefore, the ANG2 selectivity of anti-VEGF/ANGPT F(ab')2 was lost at high concentration. The anti-ANGPT2 mAb tested in the second mechanistic in vivo study bound to ANGPT2 with an affinity similar to anti-VEGF/ANGPT F(ab')2 and anti-ANGPT2 Fab, but showed no affinity for VEGF or ANGPT1.

A repeat-dose ITV GLP toxicity study (Table 4, Study 1) was performed in cynomolgus monkeys to assess the safety of anti-VEGF/ANGPT F(ab')2 prior to human clinical testing. Following 10 weeks of biweekly ITV dosing, no systemic toxicity was observed, and, ERG and FA were normal. Three animals (1 mid-dose male and 2 high-dose males) developed moderate to severe intraocular inflammation characterized by aqueous cell/flare, vitreous cell, and perivascular sheathing around the retinal blood vessels after two to three doses. This was attributed to the development of ADA against a humanized protein in these animals, and was consistent with findings observed with other ITV biologics.26,27

Following the second ITV dose, all mid- and high-dose animals demonstrated sporadic anterior segment inflammation. On clinical ophthalmic evaluation some (but not all) animals developed elevated IOP (Fig. 2). In order to control IOP and prevent eye pain in the affected animals, levobunolol and/or dorzolamide hydrochloride were administered to the eyes of animals with IOP levels more than 30 mm Hg (40% of middose and 80% of high-dose animals). These treatments were transiently effective at lowering IOP; however, IOP generally increased following the subsequent ITV doses, leading to an episodic time course of high IOP following each dose with a lowering of IOP back toward baseline and a subsequent increase in IOP after the next dose. In general, the peak IOP was observed 5 to 7 days following each dose, suggesting a delayed pharmacologic effect rather than a $C_{max}$-driven toxicity. Mean IOP spikes were 25 to 35 and 35 to 45 mm Hg in the mid- and high-dose groups, respectively, with individual animals having IOP that increased up to 55 mm Hg. Gonioscopy of eyes with increased IOP demonstrated the iridocorneal angle to be clinically open and free of inflammatory debris.

Figures 3 and 4 show the effects of anti-VEGF/ANGPT F(ab')2 administration on IOP in five representative animals (2 mid- and 3 high-dose). Pharmacologic intervention was generally required to bring IOP back to baseline; however, IOP did return to baseline without such treatment on occasion, particularly in the middose animals. Both eyes generally responded similarly, though this was not true in all animals, and may have been impacted by the development of ADA or ADA-related inflammation (Fig. 3B). A return of IOP to baseline was observed in all middose animals and most high-dose animals between doses; however, in a few animals, elevated IOP was observed prior to administration of the next scheduled dose on the days of dosing (Fig. 3C). The IOP of all animals in the recovery groups returned to baseline during the 2-month treatment-free recovery period, though in some cases, IOP-lowering treatment was used during this time.

On clinical ophthalmic evaluation some (but not all) animals demonstrated sporadic anterior segment inflammation.

### Table 4. Intravitreal Cynomolgus Monkey Study Designs

| Group                  | Test Article | Dose (mg/eye)* | ANGPT1 Inhibition† | ANGPT2 Inhibition‡ | Terminal Necropsy (n)§ | Recovery Necropsy (n)||
|------------------------|--------------|----------------|-------------------|-------------------|------------------------|-------------------|
| Study 1: 10-wk repeat-dose GLP toxicity study |
| 1 Vehicle Control      | 0            | N/A            | N/A               | 3M/3F             | 2M/2F                  |                   |
| 2 Anti-VEGF/ANGPT F(ab')2 | 0.5         | 36×            | 23,148×           | 3M/3F             | 2M/2F                  |                   |
| 3 Anti-VEGF/ANGPT F(ab')2 | 1.5         | 109×           | 69,444×           | 3M/3F             | 2M/2F                  |                   |
| 4 Anti-VEGF/ANGPT F(ab')2 | 4.0         | 290×           | 185,185×          | 3M/3F             | 2M/2F                  |                   |
| Study 2: 4-wk investigational study |
| 1 Vehicle Control      | 0            | N/A            | N/A               | 3M/3F             | 2M/2F                  |                   |
| 2 Anti-VEGF Fab        | 2            | N/A            | N/A               | 5                 | -                      |                   |
| 3 Anti-ANGPT Fab       | 2            | 312×           | 333,333×          | 3                 | -                      |                   |
| 4 Anti-ANGPT2 mAb      | 3            | N/A            | 98,039×           | 3                 | -                      |                   |

* Study 1: Test articles were administered bilaterally by ITV injection (50 μL/eye) every 2 weeks for six total doses. Study 2: Test articles were administered bilaterally by ITV injection (50 μL/eye) every 2 weeks for three total doses.
† Fold over $IC_{50}$ for ANGPT1 for test article at calculated $C_{max}$ in the eye immediately following dosing.
‡ Fold over $IC_{50}$ for ANGPT2 for test article at calculated $C_{max}$ in the eye immediately following dosing.
§ Study 1: day 72, study 2: day 35
|| Study 1 only, day 99 (1-month recovery).
When present, it was characterized by mild (1+)
} aqueous flare or cell 2 days postdose. The anterior segment inflammatory response typically resolved in these eyes by the next examination, 1 week postdose. Increased IOP was observed both in eyes without inflammation as well as in eyes with noted sporadic mild inflammation.

Several changes often associated with inflammation were observed sporadically by OCT, including retinal nerve fiber layer (RNFL) thickening, retinal vessel thickening, perivascular sheathing, and swelling around the optic nerve. Changes often associated with elevated IOP were observed and included optic disc cupping, neuroretina rim thinning, and RNFL thinning. Optic disc cupping and neuroretinal rim thinning were noted at weeks 6 and/or 10 (data not shown). Full-field ERG was normal at all time points evaluated (data not shown).

Upon histologic examination of the eyes, axonal degeneration in the optic nerves was noted among males administered 4 mg/eye and females administered 1.5 mg/eye or more (Fig. 5). Axonal degeneration was characterized by scattered clear vacuoles often contained swollen, pale eosinophilic fragmented material, degenerate myelin, and occasionally pyknotic debris. In more chronic cases, there was decreased numbers of axons, decreased diameter of the remaining axons, and pial septa thickening. Axonal degeneration was considered secondary to test article-induced high IOP and did not reverse after the 4-week recovery. Consistent with the histologic changes in the optic nerves, within the retina there was ganglion cell degeneration and loss, decreased cellularity of the inner nuclear layer, disruption of the photoreceptor cell layer with atrophy of the photoreceptor outer segments and loss of photoreceptor cell bodies. In addition, minimal-slight inflammation characterized by mononuclear cell infiltrates comprising lymphocytes, plasma cells, and macrophages was present in the eyes of all treated animals, suggestive of an immunogenic response to the humanized anti-VEGF/ANGPT F(ab')2.

Immunogenicity was assessed prestudy and on days 0, 6, 14, 28, 56, and 71. All control group animals remained ADA-negative throughout the study; by the end of the study, ADAs were observed in 9 of 10 (90%) low-dose animals, and 10 of 10 (100%) in both the mid- and high-dose groups. In general, ADAs in the low-dose group appeared earlier (earliest ADA positive observed at day 28 in the low-dose group compared with day 6 for the mid- and high-dose groups), and titers in the low-dose group were lower than in the mid- and high-dose groups; however, there was significant variability within and between groups. The low animal numbers per dose group precluded substantive analysis. The incidence of ADA did not correlate with effects on IOP. A subset of animals, with ADA to the test article, developed more persistent inflammation.

The prevailing therapeutic hypothesis of targeting the ANGPT/TIE2 pathway in AMD is that inhibition of ANGPT2, but not ANGPT1, would be beneficial in stabilizing the retinal vasculature. Therefore, the anti-VEGF/ANGPT2 F(ab')2 was engineered with approximately 650-fold selectivity for ANGPT2 (IC50 = 0.178 nM) over ANGPT1 (IC50 = 115 nM). However, because of the high local concentration of drug, even at the low dose of 0.5 mg/eye in the repeat-dose GLP toxicity study, the Cmax of anti-VEGF/ANGPT F(ab')2 is predicted to exceed the IC50 for ANGPT1 by approximately 36-fold. Based on the measured half-life of approximately 3 days for anti-VEGF/ANGPT F(ab')2, vitreous exposure likely exceeded the ANGPT1 IC50 for up to a week. While the postdose anti-VEGF/ANGPT F(ab')2 concentration in SC or the local lymphatic vessels is unknown, it is likely that both ANGPT1 and ANGPT2 were completely inhibited for significant portions of each dosing cycle at all dose levels in the repeat-dose GLP toxicity study resulting in the observed increase in IOP.

In order to further investigate the mechanism of the increased IOP seen with anti-VEGF/ANGPT F(ab')2, a follow-up cynomol...
FIGURE 3. Individual animal examples of IOP increases following bi-weekly administration of 4.0 mg/eye anti-VEGF/ANGPT F(ab’)2 in the GLP cynomolgus monkey toxicology study. (A) Animal 104511 (high-dose female) from the recovery group. (B) Animal 104492 (high-dose male) from the recovery group. (C) Animal 104490 (high-dose male) from the terminal group. Red arrows = dose administration time points. Hashed areas = duration of administration of IOP lowering treatment(s), levobunolol, and/or dorzolamide hydrochloride.

FIGURE 4. Individual animal examples of IOP increases following bi-weekly administration of 1.5 mg/eye anti-VEGF/ANGPT F(ab’)2 in the GLP cynomolgus monkey toxicology study. (A) Animal 104507 (middose female) from the recovery group. (B) Animal 104503 (middose female) from the terminal group. Red arrows = dose administration time points. Hashed areas = duration of administration of IOP lowering treatment(s), levobunolol, and/or dorzolamide hydrochloride.
FIGURE 5. Histologic comparison of the eyes and optic nerve from a control (A, C, E, and G) and high-dose (4mg/eye) anti-VEGF/ANGPT (Fab')2-treated animals (B, D, F, H). Retina (A, B; ×200; H&E). Ganglion cell degeneration (D) and loss was observed in animals treated with anti-VEGF/ANGPT (Fab')2, with decreased cellularity of the inner nuclear layer (INL) and disruption of the photoreceptor cell layer (PRL). Optic nerve (C, D; ×200; H&E). Axonal degeneration (V) was noted in the optic nerve of animals treated with anti-VEGF/ANGPT (Fab')2 (B). There was a concurrent increase in glial cells. Anterior chamber (E, F ×25; G, H ×100; H&E). The drainage angle was histologically unremarkable in all eyes examined. CB, ciliary body; C, cornea; I, iris; L, lens; T, trabecular meshwork.
A gus monkey study was conducted (Table 4, Study 2), which included the individual Fabs from the bispecific molecule (anti-VEGF Fab and anti-ANGPT Fab) along with an ANGPT2 selective antibody, anti-ANGPT2. In this study animals were dosed bilaterally ITV every 2 weeks for a total of three doses. No effects on IOP were observed in the vehicle control, anti-VEGF Fab, or anti-ANGPT2 dose groups. However, elevated IOP was observed in the anti-ANGPT Fab dose group after the second dose (Fig. 6A). To facilitate the evaluation of the natural course of the IOP effects, no IOP-lowering drugs were administered in this study. Indeed, while a slight reduction in IOP was noted in the days prior to the third dose, the IOP did not return to baseline in two of three animals, as was generally observed in the repeat-dose GLP toxicology study. Interestingly, in the animal (Animal 109837) in which IOP returned to baseline after the second dose, no increase in IOP was observed at all following the third dose (Fig. 6B). Further analysis revealed that all three animals in this dose group were ADA positive, and the animal showing a return to baseline IOP after the second dose had the highest ADA titer (Fig. 6C). It is therefore possible that the lack of response in this animal can be explained by a neutralizing effect or rapid clearance as a result of the ADAs. In support of this, serum exposure (data not shown) to the anti-ANGPT Fab was undetectable following the final (day 29) dose administration in this animal (Animal 109837), while serum exposure in Animal 109836 (which had the lowest ADA titers) was as expected. Serum exposure in Animal 109838 spiked significantly following the third dose, suggesting an inhibition of normal clearance mechanism by the ADAs in this animal. However, the neutralizing activity of these ADA was not assessed and the relevance of serum ADA to ocular ADA as well as the serum exposure to the local effects on IOP are unclear at this time.

To further elucidate the mechanism of this elevated IOP effect, AS chamber OCT was used to visualize the iridocorneal angle and trabecular meshwork in all animals in the anti-ANGPT Fab-dose group. No effects of anti-ANGPT Fab treatment were observed (data not shown); the iridocorneal angle remained open and qualitatively the trabecular meshwork and other structures appeared normal. This parallels the finding that the drainage angle was histologically unremarkable in all eyes examined. Similarly, an assessment of aqueous humor formation in all animals in this dose group showed no effect of anti-ANGPT Fab treatment (data not shown). These data suggest that the effects of anti-ANGPT Fab and VEGF/ANGPT F(ab’)2, which lead to increased IOP are not related to the rate of AHF or to the structure/function of the trabecular meshwork.

In order to determine if the effects observed following dual inhibition of ANGPT1/2 in cynomolgus monkeys were mechanistically similar to those reported following conditional genetic deletion of Angpt1 and Angpt2 in mice, a murine model was used to further characterize the effects of the anti-ANG2 antibodies on the development of SC. To examine the effect of pharmacologic ANGPT1/ANGPT2 blockade on SC development, we treated neonatal mice with anti-ANGPT or anti-ANGPT2 antibodies at P1, P3, and P5, and necropsied at P7 (Figs. 7A, 7B). By P7, SC vessels were evident in control antibody-treated mice, as shown by the co-expression of venous marker Endomucin and lymphatic marker Prox1 (Figs. 7C, 7D). In contrast, simultaneous blockade of ANGPT1

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**Figure 6.** IOP effects and potential effects of ADA on the investigational study. (A) Mean IOP levels. Red arrows = dose administration time points. *Statistical increase from baseline IOP (Dunnet’s t-test fold change from baseline, \( P < 0.05 \)). (B) Individual animal data from the anti-ANGPT Fab dose group. (C) ADA titers. Black line and circles = vehicle-control animals. Blue line and circles = Animal 109836 (anti-ANGPT Fab-treated). Red line and circles = Animal 109837 (anti-ANGPT Fab-treated), green line and circles = Animal 109838 (anti-ANGPT Fab-treated). Red arrows = dose-administration time points.
and ANGPT2 drastically impaired the formation and expansion of SC vessels, similar to genetic ablation of ANGPT1/ANGPT2 and their receptor Tie2.14,15 However, blockade of ANGPT2 alone did not affect the formation of SC, consistent with previous reports that Angpt2 is dispensable for SC development.15 While it is unknown if pharmacologic inhibition of ANGPT1/2 would have a similar effect in adult mice, taken together, these data suggest a critical role for ANGPT1 and ANGPT2 in SC development and homeostasis.

**DISCUSSION**

The role of the ANGPT/TIE2 pathway in the control of IOP was initially identified using genetic mouse models,13–15 which suggested that the ocular hypertension observed when both TIE2 ligands (ANGPT1 and ANGPT2) were ablated was due to a developmental defect or (in adult mice) impaired integrity of SC. In the current report, we have recapitulated this IOP effect in adult cynomolgus monkeys using an anti-VEGF/ANGPT F(ab')2 antibody. Axonal degeneration in the optic nerves was noted, characterized by scattered clear vacuoles, often containing pale eosinophilic cellular debris was noted upon histologic examination of the eyes (Fig. 5). Vacuolation of the optic nerve with increased glial cells, and multifocal optic nerve degeneration was characterized by scattered clear vacuoles, which often contain swollen, pale eosinophilic fragmented axons and/or myelin debris. In more chronic cases, there were decreased numbers of axons, decreased diameter of the remaining axons, and pial septa thickening. The loss of axons may lead to decreased overall diameter of the nerve. Variable glial cell proliferation and/or macrophage infiltration may occur concurrently. Follow-up studies identified the relatively nonselective anti-ANGPT arm of this molecule as the cause of this effect and confirmed that a more selective anti-ANGPT2 inhibitor does not induce an increase in IOP. These data confirm the role of ANGPT/TIE2 in control of IOP and extend the transgenic mouse work, for the first time demonstrating that the ANGPT/TIE2 axis is required for homeostasis of IOP in normal adult monkeys.

Mutations resulting in loss-of-function of the ANGPT1 or TIE2 have been found in families with primary congenital glaucoma (PCG).15,28 Through exome sequencing, Souma et al.28 identified 10 heterozygous TIE2 variants in 189 families, and these variants have been experimentally validated as loss-of-function mutants. Consistently, TIE2 haploinsufficiency in mice causes development of hypomorphic SC and ocular hypertension, demonstrating the sensitivity of TIE2 signaling strength for SC development. A follow-up study from the same group further identified three rare ANG1 variants within a cohort of 284 PCG patients.15 Functional assays and transgenic mouse studies characterized two variants as loss-of-function mutations. Importantly, no ANGPT2 variants were identified associated with PCG in the study. These results highlight the important role of ANGPT1/TIE2 signaling in regulating SC development and IOP in humans. These results also suggest that ANGPT1 inhibition alone may produce similar effects to those observed in the
These data, together with the Angpt1/2 knockout mouse inhibition is likely not related to aqueous humor production. Hydrochloride) were able to temporarily reduce IOP in the normal in all animals, and two drugs, which reduce aqueous pathways. ANG1/ANG2 and TIE2 have been reported to muscle contractility are known to impact the outflow pathway, if present, are at the molecular or cellular level. Molecular changes in trabecular meshwork and/or ciliary matrix deposition and organization and possibly SC apoptosis of endothelial cells. Integrins and these molecules are involved in cell matrix adhesion, cell migration, cell-cell adhesions, extracellular matrix deposition and organization and possibly SC endothelium. In addition, aqueous humor production was normal in all animals, and two drugs, which reduce aqueous humor production (levobunolol and/or dorzolamide hydrochloride) were able to temporarily reduce IOP in all dose groups (Table 3), and both antibodies likely completely inhibited their targets for significant periods of time. In contrast, the selective anti-ANGPT2 had no measurable binding to ANGPT1 and had no impact on IOP in cynomolgus monkeys, thereby supporting the hypothesis that dual inhibition of both ANGPT1 and ANGPT2 is required to cause an increase in IOP. While the concentrations of drug in the eye were many fold above those required to inhibit ANGPT2 immediately following dose administration, they are still relevant to the anticipated clinical doses for these inhibitors. Due to the desire to limit the number of invasive ITV injections by giving a large bolus dose, which will maintain ANGPT2 and VEGF inhibition over 4 to 6 weeks. Lower doses, while potentially sparing ANGPT1 and avoiding the IOP effects, would be impractical due to the need for biweekly (or more frequent) ITV administration. Assessments of the aqueous outflow and trabecular meshwork using anterior-chamber OCT, and gonioscopy did not reveal any structural changes to these tissues resulting from administration of the anti-ANGPT-containing antibodies. Gonioscopy of eyes with increased IOP also demonstrated the iridocorneal angle to be clinically open and free of inflammatory debris. This suggest that alterations in the outflow pathway, if present, are at the molecular or cellular level. Molecular changes in trabecular meshwork and/or ciliary muscle contractility are known to impact the outflow pathways. ANG1/ANG2 and TIE2 have been reported to affect integrins and these molecules are involved in cell matrix adhesion, cell migration, cell-cell adhesions, extracellular matrix deposition and organization and possibly SC endothelium. In addition, aqueous humor production was normal in all animals, and two drugs, which reduce aqueous humor production (levobunolol and/or dorzolamide hydrochloride) were able to temporarily reduce IOP in the affected animals, indicating that the effect of dual ANGPT1/2 inhibition is likely not related to aqueous humor production. These data, together with the Angpt1/2 knockout mouse data and our own data showing the compromised development of SC in mice treated with an antibody that blocks both ANGPT1 and ANGPT2 (but not an ANGPT2 selective antibody), support the hypothesis that ANGPT1/ ANGPT2 signaling is important for maintaining the integrity of the aqueous humor outflow in the eye. This effect appears to be reversible, as IOP levels returned to normal in the treated NHPs between doses (likely as the vitreous concentration fell below the IC50 for ANGPT1). Additionally, following cessation of anti-VEGF/ANGPT F(ab')2 treatment, the IOP of animals in the recovery groups returned to baseline. This may suggest that the defect is related to patency of the outflow pathway rather than necrosis or apoptosis of endothelial cells. SC is a unique endothelial-lined structure encircling the anterior part of the eye that is critical for delivering aqueous humor to the collecting channels and eventually the systemic vasculature. Abnormal function of the SC inner wall endothelium is likely a key factor contributing to aqueous humor outflow resistance and subsequent ocular hypertension. The endothelial cells of SC have both blood and lymphatic properties that develop in the first few weeks after birth in mice. Recent studies using genetically modified mice suggest important functions of ANGPT1/ANGPT2 signaling in SC development. In follow-up studies in mice confirmed that the effect observed in cynomolgus monkeys is similar to those reported following dual conditional genetic deletion of Angpt1 and Angpt2 in mice. This report, Angpt1 and Angpt2 were deleted at E16.5 of gestation, allowing for normal embryonic development, but producing ocular hypertension and glaucoma shortly after birth due to a compromised drainage from the eye via SC. In the current study, the effect on SC was reproduced by treating mice at P1, P3, and P5 with nonselective anti-ANGPT. In contrast, the specific anti-ANGPT2 (full-length mAb) did not affect the development of SC when administered using the same treatment paradigm. These results demonstrate that the mechanism of genetic deletion of both TIE2 ligands in mice and the pharmacologic inhibition of TIE2 signaling in cynomolgus monkeys produce the same effects on IOP. This suggests that ANGPT/TIE2 signaling is required for normal ocular drainage and homeostasis and IOP control across mammalian species. In the 10-week GLP cynomolgus monkey toxicology study, ADAs against anti-VEGF/ANGPT F(ab')2 were noted in the majority of animals. However, the presence of ADAs did not affect the IOP increase, which was sustained in all animals dosed with 1.5 mg/eye or more. However, in the 1-month nonclinical cynomolgus monkey study, the anti-ANGPT Fab increased more rapidly in all dosed animals, and in one animal the presence of ADA appeared to block the IOP effect. Further work is needed to confirm that the ADAs in this animal were able to neutralize the activity of the anti-ANGPT Fab. Dual inhibition of ANGPT1/ANGPT2 offers an intriguing potential for a nonhuman primate animal model for glaucoma research, which better preserves the integrity of the AS than current models. The present study, together with the transgenic mouse studies described here have established that dual inhibition of ANGPT1/ANGPT2 can produce ocular hypertension in both rodents and NHPs. However, unlike genetic manipulation, the use of targeted ANGPT1/ANGPT2 inhibitors brings the added benefit of producing a reversible effect on ocular hypertension, which is an advantage over many current nongenetic models, such as laser ablation or physical blockage of the trabecular meshwork. The anti-VEGF/ANGPT F(ab')2 and nonselective ANGPT Fab used in the current study are not ideal reagents, because they were not humanized as potential therapeutics, and have been shown to lead to ADA development, which can in turn cause unwanted local ocular inflammation or neutralize the pharmacologic effect of these reagents in nonclinical species. The development of species-matched dual ANGPT1/ANGPT2 inhibitors would be extremely valuable for validation of this potential glaucoma model. The possibility also exists that potentiating TIE2 signaling in SC could lead to increased aqueous outflow, though further work is required to investigate the therapeutic benefit of modulation of TIE2/ ANGPT signaling. In conclusion, we have demonstrated that dual inhibition of ANGPT1/ANGPT2, but not inhibition of ANGPT2 alone, leads to ocular hypertension and glaucomatous damage in normal adult cynomolgus monkeys. These findings confirm a role for TIE2/ANGPT signaling in the control of IOP, initially identified by transgenic mouse models. Dual inhibition of ANGPT1/ ANGPT2 should be avoided in potential antineovascular therapies targeting AMD due to potential on-target increases in IOP. Additionally, dual inhibitors of this pathway may represent key tools in the development of novel models of open-angle glaucoma.
Acknowledgments
The authors thank Paul L. Kaufman for helpful discussions, Julie Kiland for performing fluorophotometry, Doris Zane for support and overseeing the conduct of toxicology studies, Xiaoying Gao for her contribution to development/qualification of the ADA assay and sample testing, and John Lowe for development/qualification of the PK assay and sample testing.

Disclosure: E.A. Thackaberry, Ra Pharmaceuticals (E); Y. Zhou, Genentech, Inc. (E); C.L. Zuch de Zafra, Genentech, Inc. (E); V. Bantseev, Genentech, Inc. (E); C.A. Rasmussen, OcularServicesOnDemand (C); P. E. Miller, OcularServicesOnDemand (C); E. Wakshull, Genentech, Inc. (E); M. Yan, Genentech, Inc. (E); V. Rantsseev, Genentech, Inc. (E)

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