AMG 386, a Selective Angiopoietin 1/2-Neutralizing Peptibody,
Inhibits Angiogenesis in Models of Ocular Neovascular Diseases

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Supplementary Material
MATERIALS AND METHODS

Cynomolgus Monkey studies

Cynomolgus monkeys (*Macaca fascicularis*) of Vietnamese origin were housed in individual stainless steel cages; or in silanized metabolism cages for the collection of urine and feces (autoradiography study only). Animals were provided with water and Certified Primate Diet (2055C; Harlan Teklad, Madison, WI) ad libitum; or with food one to two times daily except for fasting before ocular photography and fluorescein angiography (CNV study only). Animals in the autoradiography study were acclimated to the study room for at least 1 week prior to dosing; animals in the CNV study were acclimated to laboratory conditions for at least 3 weeks prior to study initiation. All cynomolgus monkeys were observed twice daily (a.m. and p.m.) for mortality and signs of pain and distress and once daily for general health and appearance, with particular attention paid to the eyes of animals in the CNV study.

Laser-induced CNV

Ocular examinations

Animals were anesthetized with ketamine and eyes were dilated with a mydriatic agent. The adnexa and anterior portions of both eyes were examined using a slit-lamp biomicroscope. The ocular fundus of each eye was examined using an indirect ophthalmoscope. Ocular photographs were taken on the day of laser treatment (after the laser treatment) and on day 23 (day 21 postlaser), concomitant with the final fluorescein angiography (FA) interval. Color photographs were taken to document FA results and to aid in isolating laser areas for histopathologic evaluation.

On day 2, the macula of each eye underwent treatment (Comparative Ophthalmic Research Laboratories, Madison, WI) with a 532-nm diode green laser (OcuLight GL,
IRIDEX Corporation, Mountain View, CA), using a slit-lamp delivery system and a Kaufman-Wallow plano fundus contact lens (Ocular Instruments, Inc., Bellevue, WA). Laser parameters included a 75-μm spot size, 0.1 second duration, and power ranging from 400 to 650 mW. The power was initially set at 500 mW. If no hemorrhage occurred at a given spot, a second spot was placed adjacent to it using a laser intensity of 650 mW (except for the spot just temporal to the fovea, which had an initial power setting of 400 mW followed by 550 mW if there was no hemorrhage following the first spot). Ophthalmic examinations were performed once before initiation of treatment and on day 21. FA was conducted via standard methods.1-3 Within 1 minute of the fluorescein injection, a rapid series of stereo photographs of the posterior pole of the right eye were taken followed by stereo pairs of the posterior pole of the left eye. This process was then repeated at approximately 1 to 2 minutes and at 5 minutes. Between approximately 2 and 5 minutes, nonstereoscopic photographs were taken of two mid-peripheral fields (temporal and nasal) of each eye.

Evaluation of fluorescein angiograms for evidence of excessive permeability (fluorescein leakage) or any other abnormalities was performed by an investigator masked to treatment assignments according to the following grading system: Grade 1, no hyperfluorescence; Grade 2, hyperfluorescence without leakage; Grade 3, hyperfluorescence early or mid-transit and late leakage; Grade 4, bright hyperfluorescence early or mid-transit and late leakage beyond borders of treated area.1 Grade IV lesions are considered to most closely resemble the active forms of classical CNV seen in various human retinal disorders, including AMD.1

Radioanalysis and whole-body autoradiography study

Radioanalysis sample collection and preparation
Approximately 3 mL of blood was collected from each animal via the femoral vein at 1, 8, 24, 48, 72, 96, 120, and 168 hours post-dose. The first 2.5 mL were collected into tubes without anticoagulant and the remainder was collected into tubes containing sodium ethylenediaminetetraacetic acid (EDTA). After clot formation of the untreated samples, blood was centrifuged to obtain serum. Both serum and sodium EDTA-treated blood were analyzed for total radioactivity and total radioactivity after precipitation with 50% trichloroacetic acid (TCA). One animal of each sex was euthanized using Beuthanasia-D (Schering-Plough Animal Health, Boxmeer, The Netherlands) following the blood collections at 1, 24, 72, and 168 hours post-dose. From the euthanized animals, eye tissues, urine and feces (at 24-hour intervals prior to euthanasia at 72 and 168 hours post-dose), and cage debris (after excreta collections through 48 and 144 hours post-dose, respectively) were collected. Samples except for eye tissues, which were used without further preparation, were homogenized and analyzed for radioactivity in a Packard COBRA II 5003 (Waltham, MA) solid scintillation counter (SSC) for at least 5 minutes or until 1,000,000 counts. All samples were analyzed in duplicate if sample size permitted. For eye tissue (cornea, aqueous humor, lens, iris-ciliary body, retina, choroid, retinal pigmented epithelium, sclera, vitreous humor, optic nerve), a duplicate sample was analyzed first by SSC and then was precipitated with TCA prior to repeated SSC analysis.

Whole-body autoradiography

Within 20 minutes of sacrifice, carcasses were shaved and frozen in a hexane/dry ice bath for about 1 hour and then prepared for storage prior to autoradiographic analysis. Sections representing all major tissues, organs, and biological fluids were represented in sections collected at five to six levels of interest in the sagittal plane. Mounted sections were wrapped with Mylar film and exposed for 3 days on phosphorimaging screens.
Exposed screens were scanned with an Amersham Biosciences Storm phosphorimager (Amersham Biosciences).

**Murine ROP study**

**Measurement of AMG 386 Concentrations**

Concentrations of AMG 386 in the serum were measured using an enzyme-linked immunosorbent assay (ELISA) method. Human Ang2 was passively adsorbed onto a 96-well high-binding microtiter plate. After excess Ang2 was removed by washing, standards, control samples, and test samples containing AMG 386 were added to the plate. After excess unbound material was removed by washing, a horseradish peroxidase-labeled mouse monoclonal anti-human IgG1 (Fc-specific) was added, which then bound to AMG 386 captured in the previous incubation. After excess conjugate antibody was removed by washing, a substrate solution (tetramethyl benzidine, H₂O₂) was added, producing color reaction. The color reaction was stopped by adding 2N H₂SO₄ and absorbance was measured at 450–650 nm. The concentrations of AMG 386 in control and test samples were determined using a Watson LIMS comparison to a concurrently analyzed standard curve.

**In situ hybridization**

cDNA templates containing fragments from the coding sequence of Ang1, Ang2, or VEGFR2) or the 3’ untranslated region of VEGF were generated by polymerase chain reaction and were cloned into either the pCR4-TOPO vector (Invitrogen, Carlsbad, CA) or the pGEM-T vector (Promega, Madison, WI). cDNA vector constructs were linearized and ³³P-labeled riboprobes were transcribed (Table S1).
Retina sections (5 μm) were deparaffinized and hydrated through graded ethanols into DEPC-treated water. Sections were then subjected to deproteination (0.2M HCl; 10 min), proteinase K treatment (10 μg/ml; 10 min), acetylation (0.25% acetic anhydride in 0.1M triethanolamine; 10 min), dehydrated through graded ethanols, and prehybridized with hybridization buffer (300 mM NaCl, 20 mM Tris-HCl pH 8.0, 5 mM EDTA pH 8.0, 1× Denhardt’s solution, 0.2% sodium dodecyl sulfate, 10 mM dithiothreitol, 0.25 mg/ml yeast tRNA, 25 μg/ml polyadenylic acid, and 50% deionized formamide) for 4 hours in a humidified chamber at 56°C. After prehybridization, 1 × 10⁶ cpm of ³²P-labeled probe (1x hybridization buffer/10% dextran sulfate; 50 μl/slide) was added to each section, coverslipped with parafilm, and the slides incubated in a humidified chamber at 56°C for 16 hours. The slides were then washed in 4× SSC (3 M NaCl, 300 mM trisodium citrate; pH 7.0) at 55°C to remove the coverslips, followed by 2 successive washes in 4× SSC at 55°C for 5 min each, then 2 washes at 2× SSC at 55°C for 5 min each. The sections were treated with 20 μg/ml RNase A in RNase buffer (0.5 M NaCl, 10 mM Tris-HCl pH 8.0, 5 mM EDTA) at 37°C for 30 min. Following digestion, the slides were again washed through decreasing SSC concentrations (2× SSC, 1× SSC, 0.5× SSC; all 5 min each at room temperature) until reaching the final stringency of 0.1× SSC at 55°C for 30 min. Sections were then dehydrated through graded ethanols containing 300 mM ammonium
acetate, air dried and coated with Kodak NTB emulsion (Eastman Kodak, Rochester, NY; diluted 1:1 with 600 mM ammonium acetate), stored in light-tight boxes at 4°C with desiccant, and exposed for 3 weeks. Finally, the slides were developed in Kodak D19 developer (diluted 1:1 with water), fixed in Kodak fixer, and counterstained with hematoxylin and eosin.
RESULTS

Cynomolgus Monkey studies – Laser-induced CNV

Microscopic evaluation of laser sites

Direct laser-induced injury (severity) was similar across laser sites, animals, and dose groups. Similarity was demonstrated by recording all direct laser-induced changes, including the extent to which major components of the neuroretina were absent, which also documented that the actual site of laser injury was evaluated. Microscopically, direct laser-induced injury included a central area missing neuroretinal tissue, retinal pigmented epithelium (RPE), Bruch’s membrane, and choriocapillaris. Typical sequences of neuroretinal loss were observed (beginning with the outer segments of the rods and cones followed by their nuclei; then the inner nuclear layer and the ganglion cell layer). At the periphery of the laser focus the layers returned in reverse order as distance from the laser site increased. In the center of most laser sites, the ganglion cell layer remained as did a portion of the inner nuclear layer. The outer segments (of the rods and cones) and the outer nuclear layer were usually completely absent. Another indication of consistency of laser injury was the size of the area devoid of RPE, Bruch’s membrane, and choriocapillaris, which was similar across all sites, eyes, dose groups, and animals. The choroid was also altered showing occasional depressions or bulges (relative to the interior of the globe). Most bulges were due to foci of (presumed) photocoagulated melanin surrounded by (presumed) large histiocytic cells that were invariably present a laser sites.
REFERENCES

