Pharmacologic and Genetic Manipulation of MMP-2 and -9 Affects Retinal Neovascularization in Rodent Models of OIR

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Purpose. The efficacy of three matrix metalloproteinase (MMP) inhibitors with various selectivities (Ro-31-9790, AG3340, and DPC-A37668) was investigated in a rat model of retinopathy of prematurity, to examine the roles of MMP-2 and -9 in retinal neovascularization. The susceptibilities of MMP-2$^{-/-}$ and -9$^{-/-}$ mice to preretinal neovascularization were investigated in a mouse model of oxygen-induced retinopathy.

Methods. Sprague-Dawley newborn rats were exposed to alternating episodes of 50% and 10% oxygen (variable oxygen exposure) to induce retinal neovascularization. Three MMP inhibitors with various selectivity profiles were administered to variable oxygen–exposed rats via local or systemic routes. Antineovascular efficacy was determined in drug-treated versus vehicle-treated rat pups by computerized imaging of adenosine diphosphatase (ADPase)–stained retinal flatmounts. Wild-type C57BL/6J and isogenic MMP-2$^{-/-}$ and -9$^{-/-}$ mice were exposed to 75% oxygen followed by normoxia. The mice were killed immediately before or after the normoxic exposure, and eyes were either harvested for retinal dissection and flatmounting or were paraffin embedded and sectioned. Retinal vascular area and retinal neovascularization were assessed by adenosine diphosphatase staining of retinal flatmounts and by counting preretinal nuclei of hematoxylin and eosin–stained retinal sections, respectively.

Results. Ro-31-9790, AG3340, and DPC-A37668 had no effect on normal development of the rat retinal vasculature, regardless of dose or route of administration. Intravitreal injection of Ro-31-9790 (broad-spectrum) immediately after variable-oxygen exposure and 2 days after exposure resulted in 78% and 82% inhibition of retinal neovascularization, respectively. AG3340 (MMP-2- and -9-selective inhibitor) and DPC-A37668 (MMP-2-selective inhibitor) resulted in 65% and 52% inhibition, respectively, when administered by intravitreal injection immediately after variable-oxygen exposure. Intraperitoneal injection of 5, 15, and 50 mg/mL AG3340 or DPC-A37668 for 6 days after variable oxygen exposure resulted in 22% to 39% and 0% to 31% inhibition of neovascularization, respectively. AG3340 and DPC-A37668 administered by oral gavage at doses of 3, 10, or 30 mg/mL provided up to 42% and 86% inhibition of neovascularization, respectively. The average vascular areas of retinas from MMP-2$^{-/-}$ or -9$^{-/-}$ mice at postnatal day 12 were not significantly different from the wild-type control. There was a 75% ($P < 0.001$) and 44% ($P < 0.01$) reduction in preretinal neovascularization in oxygen-exposed MMP-2$^{-/-}$ and -9$^{-/-}$ mice at postnatal day 19, respectively, compared with wild-type control mice.

Conclusions. The results of this study suggest that MMP-2 plays a predominant role in retinal angiogenesis in both the mouse and rat models of oxygen-induced retinopathy. Furthermore, MMP-2 inhibition may be a viable therapeutic approach for ocular diseases characterized by retinal neovascularization. (*Invest Ophthalmol Vis Sci.* 2007;48:907–915) DOI:10.1167/iovs.06-0082

The term angiogenesis refers to the growth of new capillaries from preexisting blood vessels. The initial events of angiogenesis involve proteolytic basement membrane degradation; extracellular matrix remodeling; and endothelial cell (EC) proliferation, migration, and differentiation. The integration of these events in physiologic angiogenesis involves complex interactions among cells, growth factors, cytokines, and extracellular matrix components.[1]

Matrix metalloproteinases (MMPs) comprise a family of proteolytic enzymes of more than 20 members that are zinc and calcium dependent. Most MMPs are secreted in the inactive proenzyme form, some of them by endothelial cells of the angiogenic phenotype.[2] MMP proenzymes are activated, in part, by the plasminogen activator (PA) system, giving rise to active forms that digest and remodel the basement membrane and extracellular matrix.[1,3] Plasminogen is processed by urokinase-type plasminogen activator (uPA) in tissues, to produce plasmin. Once formed, plasmin can cleave inactive MMP proenzymes, giving rise to active forms that are regulated by tissue inhibitors of metalloproteinases (TIMPs).[4] The regulation of plasmin formation is multifactoral and tissue dependent. Tissue-type plasminogen activator (tPA) also facilitates the formation of plasmin[3,5] and is responsible for the fibrinolytic activity that promotes the dissolution of blood clots.[6] Although tPA is secreted by established vessels,[7] studies in a guinea pig corneal neovascularization (NV) model demonstrated that endothelial cells in new vessel sprouts secrete uPA exclusively (Jerdan JA et al. *IOVS* 1988;29:ARVO Abstract 109). uPA and tPA activities are rigidly controlled by plasminogen activator inhibitor (PAI)-1.[8]

MMP-2 and -9 degrade gelatin; elastin; and collagens IV (a major basement membrane component), V, VII, and X.[2] MMP-2 and -9 are most likely involved in tumor angiogenesis,[9–11] and recent studies indicate that MMP-2 and -9 are critical for NV in the posterior segment of the eye. For example, experiments

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with mouse MMP-2 deletion mutants suggest that MMP-2 plays a role in choroidal NV.[2]

Retinopathy of prematurity (ROP) is a potentially blinding disease of premature infants and results from exposure to the elevated oxygen levels used to compensate for underdeveloped lung function.[12] ROP has been linked to variable systemic oxygen levels during the course of oxygen therapy and relative retinal hypoxia after oxygen therapy.[12,13] The pathogenesis of ROP involves dysregulated angiogenesis. New vessels arise from the retinal capillary bed and penetrate the internal limiting membrane, extending into the vitreous to form "neovascular tufts," where number, size, and location of the tufts reflect the severity of disease.[14] The growth of neovascular tufts predispose affected infants to the sight-threatening complications of vitreous hemorrhage and tractional retinal detachment.[15]

Surgical intervention with laser or cryotherapy is a treatment option for ROP; however, functional outcomes are often suboptimal.[16,17] To enhance the management of ROP, the development of a pharmaceutical agent that prevents pathologic angiogenesis and simultaneously allows continued development of the intraretinal vasculature becomes an important objective.[18] In addition, successful therapies developed to manage ROP may be applicable to other ocular conditions in which NV plays a critical role.

Our laboratory has successfully developed a rat model of oxygen-induced retinopathy (OIR) that mimics human ROP by exposing newborn rat pups to alternating episodes of hyperoxia and hypoxia.[19] The advantages of this model are: (1) the retinal disease has been extensively characterized; (2) the severity of disease can be manipulated by adjusting the oxygen-exposure profiles; (3) the severity of the disease is reproducible between rats treated with the same exposure protocol; and (4) the pattern of preretinal NV is similar to that exhibited by infants in whom ROP developed.

In a previous study, intravitreal injection of recombinant PAI-1 provided efficacy against retinal NV in the rat, presumably by the direct inhibition of uPA.[20] Although therapeutic strategies directed at upstream angiogenic targets are being pursued clinically, this approach can have the disadvantage of lack of specificity, thereby, affecting a broad range of MMP activities related to non-angiogenic physiologic processes. We sought to improve potential clinical utility of proteinase inhibition by targeting MMPs that are likely to play a role in pathologic retinal angiogenesis. Therefore, the efficacy of the following compounds was evaluated in a rat model of ROP: (1) a broad-spectrum MMP inhibitor, Ro 31-9790; (2) an MMP-2- and -9- selective inhibitor, AG3340; and (3) an MMP-2-selective inhibitor, DPC-A37668. Different routes of administration and different doses were investigated. One of these compounds, AG3340, has been evaluated in human clinical trials for both oncologic and ophthalmic indications. Although early data appeared promising, oral administration studies of AG3340 in patients with subfoveal choroidal neovascularization associated with age-related macular degeneration were halted during phase II due to joint swelling and stiffness in the patients receiving the drug (Blodi BA et al. *IOVS* 2001;42:ARVO Abstract 1673).

There are uncertainties associated with the specific and/or nonspecific effects of pharmacological inhibitors tested in animal models of disease. To address these, we investigated the susceptibilities of MMP-2$^{-/-}$ and -9$^{-/-}$ mice to preretinal NV in a mouse model of OIR. The results of these experiments were compared to those obtained from the MMP inhibitor experiments conducted with the rat model of ROP in this study.

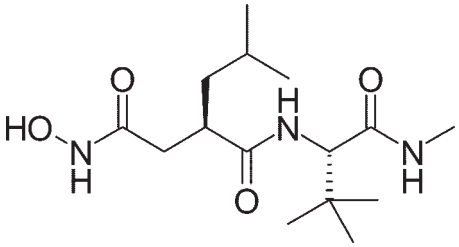

FIGURE 1. Chemical structure of Ro-31-9790.

MATERIALS AND METHODS

Oxygen Treatment

The Vanderbilt University School of Medicine Animal Care and Use Committee approved the experiments with rats and mice, and the experiments were conducted according to the principles expressed in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Rats

Randomized litters of Sprague-Dawley rats were maintained in variable-oxygen (VO) or room air (RA) environments. The VO rats were placed with mothers in infant incubators within 4 hours after birth. The rats were cycled between alternating periods of 24 hours at 50% oxygen followed by 24 hours at 10% oxygen, for 14 days. After the 14-day oxygen treatment protocol, the VO rats were moved to room air.

Mice

Seven days after birth (P7), litters of C57BJ/6L wild-type mice and isogenic MMP-2$^{-/-}$ and -9$^{-/-}$ mice were exposed to 75% oxygen for 5 days until postnatal day (P)12. Immediately after exposure, they were transferred to room air for 7 days until P19.[21]

Intravitreal Injections

Rats were anesthetized by methoxyflurane (Pitman-Moore, Mundelein, IL) inhalation and a single drop of 0.5% proparacaine (Allergan, Hormigueros, Puerto Rico) was topically applied to the cornea immediately before intravitreal injection. During all intravitreal injections, the globe was penetrated retinally, posterior to the ora ciliaris, with a 30-gauge needle with a 19° bevel and a 10-μL syringe (Hamilton Co., Reno, NV). The needle was advanced to the posterior vitreous while a steep angle was maintained to avoid contact with the lens. The injection bolus (5 μL) was delivered near the trunk of the hyaloid artery proximal to the posterior pole of the retina. After injection, a topical antibiotic suspension (neomycin and polymyxin B sulfates and gramicidin; Monarch Pharmaceuticals, Bristol, TN) was applied. Noninjected eyes were also treated with topical proparacaine and antibiotic to control for the potential of these agents to influence retinal vessel growth.

Drug Treatment

Ro 31-9790: a Broad-Spectrum MMP Inhibitor. Eyes from VO rats were injected with 5 μL of Ro 31-9790 (Roche Diagnostics Corp., Indianapolis, IN) at a 150-μg dose immediately on removal from the oxygen-exposure chamber, or 2 days after transfer to room air, which is the time of peak VEGF expression (hereafter referred to as days 14(0) and 14(2), respectively). The dose was determined from a preliminary, dose–response experiment (neovascular areas: vehicle, 2.17 mm^2; 0.03 mg/mL, 1.90 mm^2; 0.3 mg/mL, 1.63 mm^2; 3.0 mg/mL, 1.0 mm^2; and 30.0 mg/mL, 0.5 mm^2). The chemical structure of Ro 31-9790 is shown in Figure 1 with K_i shown in Table 1 (Walker K, Roche Diagnostics, personal communication, 2005). Control eyes from VO rats were either not injected or were injected with vehicle (0.2% carboxymethylcellulose [CMC] and 0.01% Tween 20 [both Sigma-Aldrich, St. Louis, MO]) on 14(0) or 14(2).

TABLE 1. K_i Values for the MMP Inhibitors

Compound	K_i (nM)			Selectivity for MMP-2 over MMP-9 (*x*-fold)
	MMP-1	MMP-2	MMP-9	
Ro 31-9790	3.0	5.2	10.4	2.0
AG 3340	8.3	0.05	0.26	5.2
DPC-A37668	6500.0	0.48	255	531.3

AG3340: an MMP-2- and -9-Selective Inhibitor. Eyes from the VO rats were injected with 5 μL of AG3340 (Alcon Laboratories, Inc., Fort Worth, TX) at a dose of 150 μg on 14(0). The control eyes were either not injected or were injected with vehicle (0.2% CMC; 0.1% Tween 20).

Drug doses administered by intraperitoneal (IP) injections and oral gavage were calculated on a per kilogram body weight basis. VO rats received IP injections of either vehicle (0.4% CMC) or AG3340 at doses of 5, 15, or 50 mg/kg. IP injections were administered daily for 6 days (14(0)–14(5)). Some VO rats were not treated and served as control subjects.

For the oral gavage study, VO rats received either vehicle (0.4% CMC) or AG3340 at doses of 3, 10, or 30 mg/kg twice daily for 6 days (14(0)–14(5)). Some VO rats were not treated to serve as controls. The chemical structure and MMP selectivity of AG3340 are presented in Figure 2 and Table 1 (Walker K, personal communication, 2005).

DPC-A37668: an MMP-2-Selective Inhibitor. DPC-A37668 (Bristol-Myers Squibb Company, Princeton, NJ) or vehicle was administered by intravitreal injection (vehicle; 0.2% CMC and 0.1% Tween 20), intraperitoneal injection (vehicle; 0.4% CMC), and oral gavage (vehicle; 0.4% CMC) at identical doses and times, as described for AG3340. Again, some VO rats served as the nontreated control. The chemical structure and MMP activity of DPC-A37668 is presented in Figure 3 and Table 1 (Bingaman D, Alcon Research Laboratories, personal communication, 2005).

Quantification of Retinopathy

VO rats were euthanatized by decapitation at P20, (i.e., 6 days after removal to room air, or 14(6)). After hyperoxia, mice were either euthanatized by decapitation on P12 or P19 after 7 days of normoxia. The eyes of the rats and mice were enucleated, and the neural retinas were dissected and placed in CMF-PBS with 37% formaldehyde solution (Fisher Scientific, Fair Lawn, NJ) also referred to as 10% neutral-buffered formalin (NBF), overnight at 4°C. The vasculature of retinas were stained by a histochemical method for detecting ADPase, according to a previously described method[22] adapted for use herein.[23,24]

Images of ADPase-stained retinas were digitized, captured, and displayed at 20× magnification. The total retinal area and the retinal area containing blood vessels was traced on the monitor face with an interactive stylus pen (FTG Data Systems, Stanton, CA).[18] The number of pixels within this area was converted to square millimeters. Measurements of this parameter were recorded.

To determine the effect of the various treatments on pathologic angiogenesis, we assessed the extent of retinal NV in flattened rat retinas stained for adenosine diphosphatase (ADPase) activity. Representative retinal flatmounts of vehicle and drug-treated rats dosed with DPC-A37668 by oral gavage are shown in Figure 4. Images of ADPase-stained retinas were digitized, captured, and displayed at 65× magnification. Vessel tufts were then outlined directly on the monitor face with the stylus pen. The pixels contained within an encircled area were counted, the total number of pixels from all areas was summed, and the result was converted to square millimeters. The preretinal nature of the tufts was confirmed by simultaneous assessment with a microscope at 200× magnification, using the plane of focus. This method of estimation correlates well ($r^2 = 0.947$) with the clock hour method of estimation[19] and yields normally distributed data that allow statistically significant differences between treatment groups to be determined by analysis of variance.

Preretinal NV in mice was evaluated according to the method described by Smith et al.[21] Transverse meridianal retinal sections were prepared from paraffin-embedded eyes and stained with periodic acid-Schiff (PAS) and hematoxylin. Preretinal NV was assessed by counting nuclei within the vitreous cavity of sections that were systematically chosen based on the distance from the optic nerve.

Gel Zymography

MMP-2$^{-/-}$, MMP-9$^{-/-}$, and wild-type mice were exposed to the oxygen model described earlier. Mice were killed at days P12 and P19, and two retinas were pooled for these mice as well as age-matched control mice raised in room air. The two retinas were then homogenized in 150 μL of extraction buffer (40 mM Tris-HCl, 110 mM Tris base [pH. 7.4], 150 mM NaCl, 5 mM CaCl, 5 mM $MgCl_2$, and 1% Triton X-100) before flash freezing. Samples were thawed and centrifuged at 20,800*g* for 8 minutes at 4°C. Protein concentration was measured in all samples with the BCA protein assay kit (Pierce, Rockford, IL), and an equivalent volume of each was affinity purified with an 8:1 ratio of sample volume to gelatin Sepharose 4B beads (GE Healthcare, Piscataway, NJ) by incubation at 4°C for 1 hour, with rocking. Samples were eluted in 40 μL of 2× zymogram sample buffer (Bio-Rad, Hercules, CA) plus 10% dimethylsulfoxide (DMSO). A 20-μL aliquot of each sample was loaded on a 10-well, 10% gelatin zymography gel (Ready Gel; Bio-Rad) with appropriate markers and controls. The gel was run for 90 minutes at 100 V in 1× Tris-glycine-SDS (20 mM Tris base, 200 mM glycine, 3 mM SDS). After incubation with shaking at 25°C in 1× zymogram renaturation buffer (Bio-Rad; 2.5% Triton X-100) for 45 minutes, the gel was left overnight (16–20 hours, optimally) at 37°C in 1× zymogram development buffer (50 mM Tris-HCl [pH 7.5], 200 mM NaCl, 5 mM $CaCl_2$, and 0.02% Brij-35; Bio-Rad). The gel was stained for 20 minutes in Coomassie blue stain (0.5% Coomassie blue R-250, 40% methanol, and 10% acetic acid in distilled water), rinsed briefly in

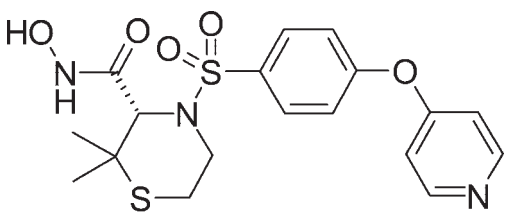

FIGURE 2. Chemical structure of AG3340.

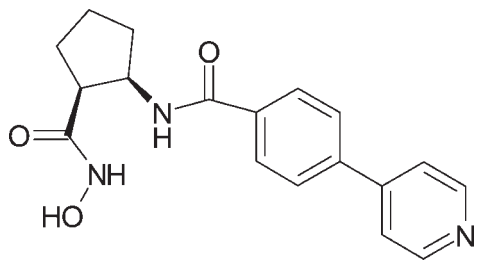

FIGURE 3. Chemical structure of DPC-A37668.

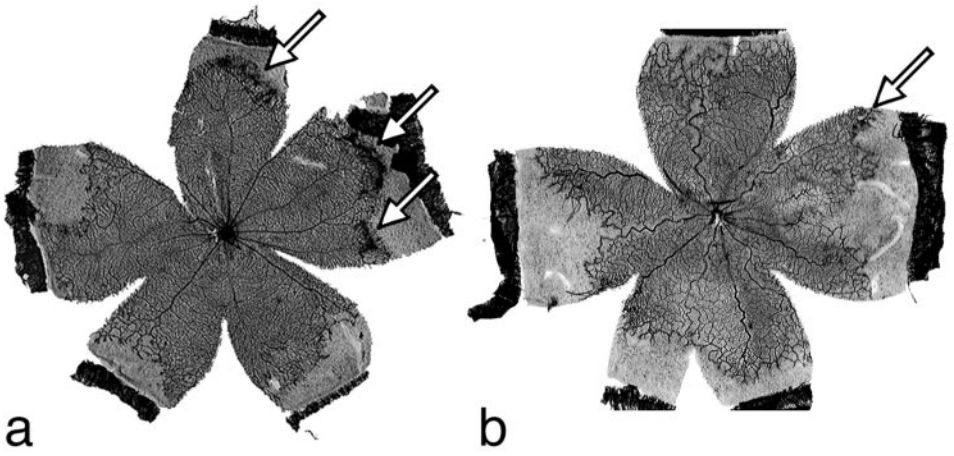

FIGURE 4. Representative retinal flatmounts from VO rats dosed with (**A**) vehicle and (**B**) DPC-A37668 by oral gavage. *Arrows:* areas of neovascularization.

distilled water, and then destained for up to 2 hours in 40% methanol plus 10% acetic acid. Zones of clearing that corresponded to the presence of proteinases in the gel were quantified by using image-analysis software (ImageJ; NIH, Bethesda, MD). The data are expressed as pixels per microgram protein (relative gelatinase activity) (see Table 5).

Gelatinase Activity Assay

On removal from oxygen, rat pups were given an intraocular injection with one of the MMP inhibitors or vehicle and then killed the following day. Retinas from these animals were collected, and the protein was isolated in a lysis buffer solution (150 mM NaCl, 50 mM Tris-HCl, 1 mM sodium orthovanadate, 1% Triton X-100, 0.1% SDS, and EDTA-free proteinase inhibitor cocktail [Complete Mini; Roche]). The proteins were then assayed for MMP gelatinase activity with an assay kit (Chemicon, Temecula, CA).

Statistical Analysis

Statistically significant differences in average MMP activities and average vascular and neovascular areas between the treatment groups and control groups were determined by analysis of variance with a Bonferroni/Dunn post hoc procedure. $P \leq 0.05$ was considered significant.

RESULTS

Ro-31-9790: a Broad-Spectrum MMP Inhibitor

Eyes from VO rats injected with Ro 31-9790 at a dose of 150 μg on 14(0) or 14(2) showed a 78% or 82% reduction, respectively, in average retinal NV on 14(6) when compared with vehicle-injected control eyes (Table 2). There was no significant difference in average retinal neovascular areas between noninjected control eyes and vehicle-injected eyes at 14(6) for vehicle injections at 14(0); however, there was a significant difference between noninjected control eyes and vehicle-injected eyes at 14(6) for vehicle injections at 14(2) ($P < 0.01$).

There was no significant difference in average retinal vascular area between Ro 31-9790-injected, vehicle-injected, and noninjected eyes at 14(6) for either of the injection times. These data are summarized in Table 2.

AG3340: an MMP-2- and -9-Selective Inhibitor

Intravitreal injection of VO rats with AG3340 at a dose of 150 μg on 14(0) showed a 65% reduction in average retinal neovascular area at 14(6) when compared with vehicle-injected control eyes (Table 3). There was no significant difference in average retinal neovascular area between the noninjected and vehicle-injected eyes. There was also no significant difference in average retinal vascular area between AG3340-injected, vehicle-injected, and noninjected eyes at 14(6).

Intraperitoneal injection of VO rats with AG3340 daily for 6 days (14(0) through 14(5)) at doses of 5, 15, or 50 mg/mL resulted in no significant differences in average body mass and no significant reduction in average retinal vascular areas when compared with VO rats injected with vehicle by the same route, or with noninjected rats. There was also no significant difference in average retinal vascular area and average body mass between the noninjected and vehicle-injected groups. The average neovascular areas of the drug-injected groups on 14(6) correspond to a 22%, 35%, and 39% inhibition of the vasoproliferative response to the low, medium, and high doses, respectively, compared with vehicle-injected groups. No difference in average retinal neovascular area was observed between the vehicle-injected group and the noninjected group.

Administration of AG3340 by oral gavage twice daily for 6 days (14(0) through 14(5)) at doses of 3, 10, or 30 mg/mL to VO rats resulted in no significant differences in average body mass or average retinal vascular area when compared with

TABLE 2. Summarized Data for the 150-μg Intravitreal Injection of Ro 31-9790 into Rats

Treatment	*n*	Treatment Day	Area of Neovascularization Mean ± SD (mm^2)	% Inhibition	*P**	Vascular Area (% of Total)
None	12	—	2.5 ± 0.7	—	—	73
Vehicle	13	14(0)	2.3 ± 0.6			70
Ro 31-9790	13	14(0)	0.5 ± 0.5	78	<0.0005	70
Vehicle	8	14(2)	1.7 ± 0.5			78
Ro 31-9790	8	14(2)	0.3 ± 0.4	82	<0.0005	79

* Compared with vehicle.

TABLE 3. Summarized Data for the Treatment of Rats with AG3340

Treatment	*n*	Area of Neovascularization Mean ± SD (mm^2)	% Inhibition	*P**	Vascular Area (% of Total)
Intravitreal injection					
None	12	2.3 ± 1.2	—	—	72
Vehicle	12	1.8 ± 0.9			74
AG3340 (150 μg)	12	0.8 ± 0.5	65	<0.005	70
Intreperitoneal					
None	7	2.5 ± 1.0	—	—	75
Vehicle	8	2.3 ± 0.5	—	—	75
5 mg/mL AG3340	7	1.8 ± 0.8	22	NS	77
15 mg/mL AG3340	5	1.5 ± 0.4	35	<0.05	73
50 mg/mL AG3340	5	1.4 ± 0.6	39	<0.05	78
Oral gavage					
None	8	2.6 ± 1.2	—	—	73
Vehicle	6	2.6 ± 0.8	—	—	76
3 mg/mL AG3340	6	2.5 ± 1.4	4	NS	72
10 mg/mL AG3340	8	2.2 ± 0.8	15	NS	71
30 mg/mL AG3340	8	1.5 ± 0.7	42	<0.02	72

* Compared to vehicle.

vehicle and untreated groups. There was also no significant difference in average retinal vascular area and average body mass between the vehicle and untreated groups. The average neovascular areas of the drug-treated groups at 14(6) corresponded to a 4%, 15%, and 42% inhibition of the vasoproliferative response for the low, medium, and high doses, respectively, compared with the vehicle group. There was no significant difference in average retinal neovascular area between the untreated and the vehicle group. These data are summarized in Table 3.

DPC-A37668: an MMP-2-Selective Inhibitor

Intravitreal injection of VO rats with DPC-A37668 at a dose of 150 μg on 14(0) provided a significant 52% inhibition of the vasoproliferative response, compared with vehicle-injected eyes. There was also a significant difference in average retinal neovascular area between the noninjected and vehicle-injected eyes ($P < 0.05$). Intravitreal injection of DPC-A37668 on 14(0) did not result in a significant difference in retinal vascular area between groups of drug-injected, vehicle-injected, and noninjected eyes (Table 4).

Intraperitoneal injection of DPC-A37668 daily for 6 days (14(0)–14(5)) at doses of 5, 15, or 50 mg/mL resulted in no significant reduction in average retinal vascular area and no significant difference in average body mass when compared with the vehicle-injected and noninjected groups. There was also no significant difference in retinal vascular area and average body mass between the noninjected and the vehicle-injected groups. There was a 17% or 31% inhibition of the vasoproliferative response for the groups receiving the 15 or 50 mg/mL doses, respectively, when compared with the vehicle-injected group. The low dose had no effect on the vasoproliferative response, and there was no significant difference in the average retinal neovascular area between the noninjected and the vehicle-injected groups.

DPC-A37668 administered by oral gavage twice daily for 6 days (14(0)–14(5))at doses of 3, 10, or 30 mg/mL resulted in no significant reduction in average retinal vascular area and no significant differences in average body mass when compared with the vehicle and the nontreated groups. There was also no difference in average retinal area and average body mass between the nontreated and the vehicle group. The retinal neo-

TABLE 4. Summarized Data for the Treatment of Rats with DPC-A37668

Treatment	*n*	Area of Neovascularization Mean ± SD (mm^2)	% Inhibition	*P**	Vascular Area (% of Total)
Intravitreal injection					
None	8	2.8 ± 0.5	—	—	68
Vehicle	10	2.3 ± 0.6	—	—	74
DPC-A37668 (150 μg)	10	1.1 ± 0.4	52	<0.005	72
Intraperitoneal injection					
None	7	2.6 ± 1.0	—	—	75
Vehicle	9	2.6 ± 0.8	—	—	74
5 mg/mL DPC-A37668	8	2.6 ± 0.9	0	NS	75
15 mg/mL DPC-A37668	5	2.1 ± 0.5	17	NS	73
50 mg/mL DPC-A37668	5	1.8 ± 0.4	31	<0.05	74
Oral gavage					
None	7	2.2 ± 1.0	—	—	70
Vehicle	9	2.1 ± 0.7	—	—	69
3 mg/mL DPC-A37668	7	2.0 ± 0.9	5	NS	69
10 mg/mL DPC-A37668	7	1.4 ± 0.9	33	<0.05	67
30 mg/mL DPC-A37668	9	0.3 ± 0.3	86	<0.0005	71

* Compared to vehicle.

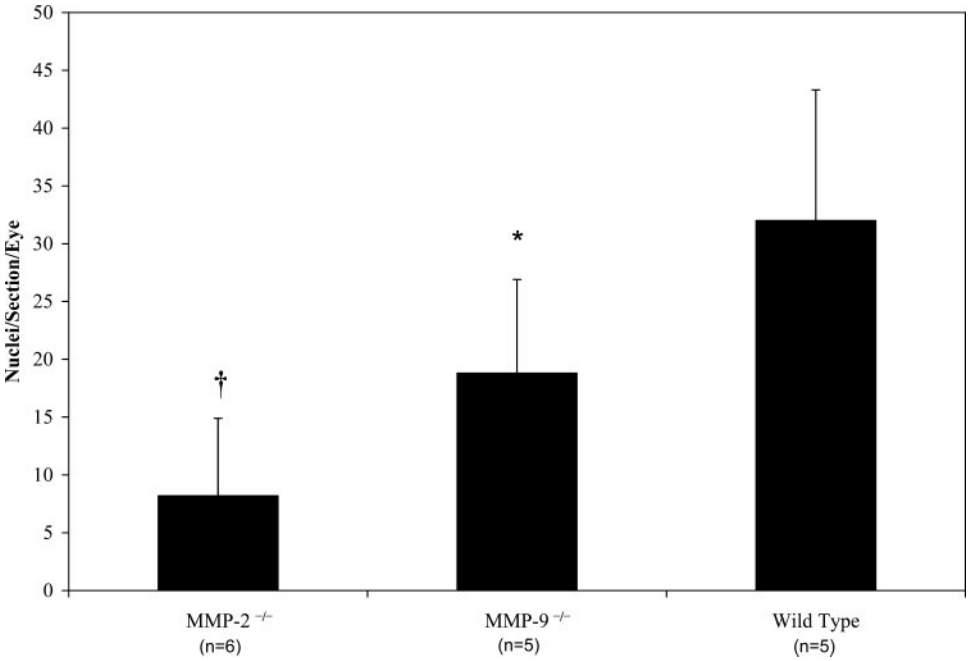

FIGURE 5. Prenuclear counts of oxygen-treated mice (mean ± SD). *$P <$ 0.01, †$P < 0.001$.

vascular areas of the groups receiving the 3, 10, or 30 mg/mL doses corresponded to a 5%, 33%, or 86% inhibition of the vasoproliferative response, respectively, when compared with the vehicle group. There was no significant difference in the average retinal neovascular area between the untreated group and the vehicle group. These data are summarized in Table 4.

MMP-2$^{-/-}$ and -9$^{-/-}$ Mice

The average percentage of vascular area (mean ± SD) in ADPase-stained mouse retinas at P12 was: (1) C57BJ/6L wild type ($n = 5$), 78.03% ± 4.52%; (2) MMP-2$^{-/-}$ ($n = 12$), 78.67% ± 3.52%; and (3) MMP-9$^{-/-}$ ($n = 4$), 78.91% ± 4.52%. There were no significant differences in percentage of avascular area between any of the groups. Preretinal NV on P19, as measured by preretinal nuclei counts, is reported as follows: (mean ± SD): (1) wild type C57BJ/6L ($n = 5$), 32.0 ± 11.3; (2) MMP-2$^{-/-}$($n = 6$), 8.2 ± 6.7 ($P < 0.001$); (3) MMP-9$^{-/-}$ ($n = 5$), 18.8 ± 8.1 ($P < 0.01$). MMP-2 and -9 gene deletion resulted in a 75% and 44% reduction in disease, respectively (Fig. 5).

The zymography of the gelatinase-deficient mice demonstrated that there was indeed a compensatory effect of the nondeficient MMP in room air mice. However, in the oxygen-treated mice, any compensatory effect was absent or greatly reduced (as seen by the difference in MMP-2 of the MMP-9-deficient mice at P12 compared with the wild-type mice, which resolved by P19), strengthening support of the notion that the observed graded neovascular response of MMP-2 vs. MMP-9 nonknockouts is directly related to the deficient MMP. In these zymograms, activity of the gelatinase is indicated by the amount of the lower-molecular-weight form of the enzyme. Both of the genetically altered strains as well as the wild-type demonstrated an increase in gelatinase activity when comparing oxygen-treated animals with room-air raised animals. The data are summarized in Table 5 No MT1-MMP (MMP-14) levels were detected.

TABLE 5. Zymographic Analysis of MMP-2 and -9

	MMP-2		MMP-9	
Treatment	**72 kDa**	**62 kDa**	**92 kDa**	**84 kDa**
Wild type				
Room air raised P12	4.10 ± 1.5	ND	6.95 ± 2.1	ND
Room air raised P19	4.56 ± 1.8	ND	8.70 ± 1.3	ND
Oxygen treated P12	6.25 ± 2.5	1.38 ± 0.5	9.80 ± 0.3	0.66 ± 0.3
Oxygen treated P19	9.19 ± 1.6	4.66 ± 2.4	8.56 ± 0.7	0.74 ± 0.4
MMP-2$^{-/-}$				
Room air raised P12	ND	ND	13.14 ± 1.8*	0.39 ± 0.1*
Room air raised P19	ND	ND	15.62 ± 1.2*	0.48 ± 0.1*
Oxygen treated P12	ND	ND	10.89 ± 0.9	0.61 ± 0.1
Oxygen treated P19	ND	ND	9.02 ± 1.7	0.90 ± 0.2
MMP-9$^{-/-}$				
Room air raised P12	7.30 ± 0.9*	2.71 ± 1.0*	ND	ND
Room air raised P19	8.09 ± 1.7*	2.43 ± 1.0*	ND	ND
Oxygen treated P12	7.19 ± 0.5	3.67 ± 0.9*	ND	ND
Oxygen treated P19	6.95 ± 1.3	4.15 ± 1.4	ND	ND

All data ($n = 4$) are expressed as pixels/microgram retinal protein. ND, activity was below the level of detection

* Significantly different from the same protein in Wild Type retinas at $P \leq 0.05$.

Discussion

Multicomparisons of the control and treatment groups shown in Tables 2, 3, and 4 indicated no appreciable differences in average intraretinal vascular areas. These observations suggest that all the compounds tested in this study, regardless of the MMP selectivity or specificity and route of administration, had no relevant effect on the normal retinal vascular development, as seen by the measurement of vascular areas. Systemic administration of AG3340 or DPC-A37668 had no significant effect on weight gain when compared with the corresponding vehicle and untreated groups, indicating no toxicity within the dose range indicated (Tables 2–4).

Intravitreal injection of the Ro-31-9790 *vehicle* at day 14(2) and the DPC-A37668 *vehicle* at day 14(0) produced a significant reduction in retinal neovascular area when compared with eyes from VO rats that were not injected ($P < 0.01$ and $P < 0.05$, respectively). A consistent reduction in retinal neovascular area was observed for the rest of the intravitreal *vehicle* injection groups in this study; however, statistical significance was not achieved when compared with noninjected control animals. Presumably, this antiangiogenic effect is related to healing of the wound created by the needle puncture. Studies in our laboratory have shown that needle puncture of the globe upregulates several angiostatic proteins in the retina that may be responsible for the attenuation of the vasoproliferative response.[25]

Intravitreal injection of the broad-spectrum inhibitor Ro-31-9790 at day 14(0) or 14(2) produced a relatively small difference in the percentage of inhibition of NV between the two injection times. These data suggest that MMP activity is a critical factor in angiogenesis for at least 2 days after oxygen exposure. Intravitreal injection of the MMP-2- and -9-selective inhibitor AG3340 at day 14(0) resulted in a 65% inhibition of NV. The inhibition constants (K_i, Table 1) of Ro-31-9790 for MMP-2 and MMP-9 are 5.2 nM and 10.4 nM compared with 0.05 nM and 0.26 nM for AG3340, respectively (Walker K, personal communication, 2005; Bingaman D, personal communication, 2005). The relative magnitudes of K_i suggest an approximate 100- and 40-fold greater inhibition of MMP-2 and -9, respectively, by AG3340 in vitro. The relative magnitudes of K_i and the modest decrease in percentage inhibition in retinal NV obtained for intravitreal injection of Ro-31-9790 from 78% to 65% for AG3340, suggests that the majority of proangiogenic MMP activity is contributed by MMP-2 and -9. Although broad MMP inhibition may explain the increased inhibition observed after a single intravitreal injection of Ro-31-9790, it is also possible that the compound is acting through an MMP-independent mechanism and/or has increased bioavailability. Intravitreal injection of the MMP-2-selective inhibitor DPC-A37668 (150 μg) at day 14(0) (K_i = 0.48 nM; Bingaman D, personal communication, 2005) produced a 52% inhibition compared with the vehicle-injected control. When viewed in light of the similar results with AG3340, this finding further suggests that MMP-2 plays a more dominant role than MMP-9 in retinal NV in this model. Another explanation for the reduced inhibition after intravitreal DPC-A37668 may be related to the 10-fold increase in K_i and/or limited bioavailability of DPC-A37668 compared with AG3340.

Intraperitoneal injection of AG3340 or DPC-A37668 showed a dose-dependent increase in efficacy against retinal NV (Tables 2, 3). However, inhibition at the highest doses of intraperitoneal AG3340 and DPC-A37668 (39% and 31%, respectively) were both substantially lower than that obtained after intravitreal injection of these compounds (65% and 52%, respectively). These observations may be related to lower bioavailability to sites of retinal angiogenesis after intraperitoneal administration than with intravitreal injection at these doses. The percentage inhibition of NV at each dose of DPC-A37668 was lower than that for AG3340. As previously discussed in the context of intravitreal injection, these data may be accounted for in terms of MMP-2 selectivity versus MMP-2 and -9 selectivity, relative K_i and difference in bioavailability between AG3340 and DPC-A37668 administered by intraperitoneal injection or a combination of these parameters.

Administration of AG3340 and DPC-A37668 by oral gavage produced a dose-dependent increase in the percentage of inhibition of NV compared with vehicle control. The percentage of inhibition of NV at the highest dose of AG3340 was substantially lower than that obtained by intravitreal injection of this drug, again suggesting limited bioavailability by this route of administration at the doses tested. However, administration of the highest dose of DPC-A37668 gave a percentage of inhibition substantially higher than that obtained by intravitreal or intraperitoneal injection and comparable to that obtained by intravitreal injection of the broad-spectrum inhibitor Ro-31-9790. This suggests that high levels of bioavailable drug can be obtained at sites of retinal angiogenesis when this drug is repetitively administered by oral gavage; moreover, bioavailability may be a dominant factor because the in vitro MMP-2 inhibition constant is 10-fold higher than that of AG3340. Because DPC-A37668 is MMP-2-selective, the relatively robust inhibition of NV suggests the central, perhaps exclusive, role of MMP-2 in the retinal angiogenic process in this disease model.

In a previous study, intravitreal injection of PAI-1 and vehicle into the eyes of VO rats resulted in a strong induction of the latent and active forms of MMP-9 as measured by analysis of zymograms[20]; yet, these eyes showed the lowest degree of retinal NV relative to noninjected eyes with greater disease. Needle puncture of the globe may be responsible for this induction of MMP-9.[20] Injection of PAI-1 demonstrated efficacy with respect to inhibition of retinal NV, and the activated form of MMP-2 in PAI-1-injected eyes was only one fourth that measured in noninjected eyes of VO rats. These observations, as well as those in other studies,[26–30] suggest a more prominent role for MMP-2 in angiogenesis.

Although there are well-documented differences in the pathologic features of the rat ROP and mouse OIR models, preretinal NV is a common component of the pathogenesis in both. When testing pharmacological inhibitors in animal models of disease, inherent ambiguities are associated with specific versus nonspecific effects. To resolve these ambiguities, we tested the susceptibilities of MMP-2$^{-/-}$ or -9$^{-/-}$ mice to the development of preretinal NV in a mouse model of OIR. MMP-2 deficiency produced a greater reduction in preretinal NV (75%) than did MMP-9 deficiency (44%). A compensatory response of the nondeficient gelatinase in the genetically altered mice was found in the room-air raised animals. This compensation may have been absent or reduced in the oxygen-treated animals because of a maximum activity of these enzymes under the OIR conditions. However, even with a compensation of MMP-2 in the MMP-9-deficient mice at P12, this compensation did not overcome the removal of MMP-9, which resulted in a significant decrease in NV compared with wild type. This decrease was not as large as the decrease in the MMP-2-deficient mice, further showing the importance of MMP-2 over that of MMP-9 in this model. Our rat experiments with pharmacological MMP inhibitors of variable selectivity and specificity also suggest that the dominant MMP activity in preretinal NV arises from MMP-2. Hence, our rat and mouse findings correlate well, and they point to the importance of MMP-2 activity in preretinal NV. However, a previous report indicated that an MMP-9$^{-/-}$ strain with a C57BJ/6L genetic background showed no difference in

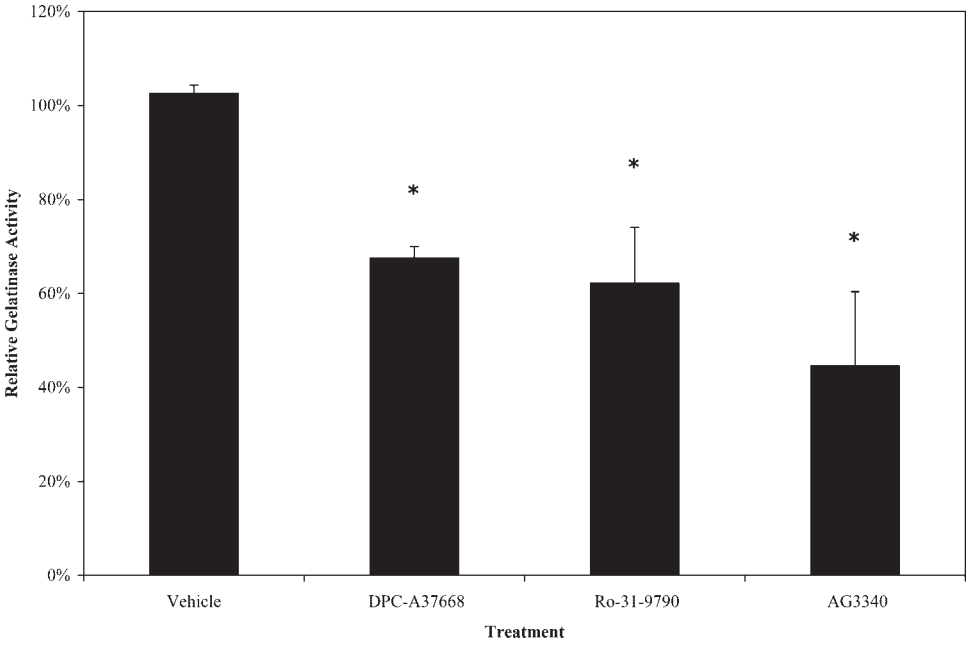

FIGURE 6. Effect of MMP inhibitor treatment on gelatinase activity levels 1 day after injection. $^*P < 0.01$.

preretinal NV compared with the wild-type.[31] The MMP-9$^{-/-}$ strain tested in our work is independent from the strain used by Ohno-Matsui et al.,[31] and the differences in susceptibility to OIR may be related to genetic variance resulting from differences in genome manipulation. In addition, a report by Sarman et al.[32] indicated no pathologic retinal angiogenic impairment in MMP-2-deficient mice. This direct discrepancy is possibly the result of the mixed genetic background of their mice containing the 129 strain, which is known to have increased susceptibility to neovascularization.[33] Also, their method of fluorescein-dextran infusion would not have detected much of the neovascular growth lacking patent, vascular lumina, which would have been shown by our ADPase staining.[34] Without optimal assessment of neovascularization, much of the difference in wild-type and gelatinase-deficient mice could go unnoticed.

We did not measure any pharmacokinetic parameters for the drugs that were tested in this study. However, even with the uncertainties associated with bioavailability and the assumptions associated with correlating in vivo inhibition with in vitro K_i data,[35,36] it is plausible that the inhibition of MMP activity was directly responsible for the efficacy demonstrated in these experiments. Furthermore, the data presented in the present study suggest that specific inhibitors of MMP-2 may be sufficient to block a substantial proteolytic component of retinal NV in the rat. MMP-2-specific inhibition would be advantageous, as other MMP activities are likely to be required for the developing retina.[20] The bioavailability of the drugs given as intravitreal injections should be nearly complete because of the drugs' injection directly into the vitreous. The vitreous is not a barrier even to large proteins, meaning these molecules would have no barrier in their effects on the retina. The best test of this effect would be through zymography of the enzymes to see the inhibition of the gelatinase activity; however, the separation techniques of gel zymography isolate the drug from the enzymes, preventing this inhibition. Therefore, an MMP gelatinase activity assay (Chemicon), specifically designed to measure the effect of MMP inhibitors on biological samples, was used. This showed a 35%, 40%, and 58% reduction in gelatinase activity in comparison to vehicle-treated levels after treatment with DPC-AA3768, Ro-31-9790, or AG3340, respectively, as shown in Figure 6. In addition, despite the possibility of an MMP-independent pathway's being inhibited in this process, both the genetic and pharmacologic manipulation of the MMPs rendered similar results, strongly supporting the bioavailability of the drugs and pharmacologic inhibition of the gelatinases in vivo.

VEGF is a potent endothelial cell mitogen, and it induces endothelial cell differentiation. Both of these bioactivities have crucial roles in angiogenesis. As a result, VEGF, VEGF receptors, and downstream signaling intermediates have received considerable attention as chemotherapeutic targets.[37–41] In this study, we targeted MMP activity (extracellular matrix digestion) that is intrinsically linked to endothelial cell migration, an early event in the angiogenic process. It is likely that targeting only one component of the angiogenic process in the pathogenesis of ROP will not provide sufficient chemotherapeutic potential. Combination therapies targeting multiple components of tumor angiogenesis have been successful, and a similar approach may be useful for ROP and other ocular conditions with an angiogenic component.

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