Inhibition of Pseudomonal Ulceration in Rabbit Corneas by a Synthetic Matrix Metalloproteinase Inhibitor

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Purpose. To evaluate the effect of the synthetic matrix metalloproteinase inhibitor, Galardin, on proteases produced by Pseudomonas aeruginosa (PA) and on a rabbit model of Pseudomonas keratitis.

Methods. Protease activities of culture broths from Pseudomonas strains PA-28 and W-186 were characterized in vitro by gelatin zymography and by digestion of Azocasein in the presence and absence of Galardin and the serine protease inhibitor, aprotinin. In a noninfectious in vivo experiment, sterile PA culture broth from W-186 was injected intrastromally into rabbit corneas that were treated topically with Galardin or vehicle, then evaluated clinically and histologically. In an infectious in vivo experiment, rabbit corneas were injected with washed PA-28, then treated topically with Galardin or vehicle and clinically scored.

Results. Gelatin zymography of culture broth from W-186 and PA-28 detected two proteases that were both inhibited by Galardin. Galardin reduced the digestion of Azocasein by both PA culture broths by 99%, whereas aprotinin did not significantly reduce the protease activity of PA-28 conditioned broth. Intrastromal injection of sterile W-186 culture broth caused rapid corneal destruction that was prevented by topical treatment with Galardin. Intrastromal injection of washed PA-28 bacteria resulted in progressive corneal melting that was significantly (P < 0.005) delayed, but ultimately not prevented, by topical treatment with Galardin.


Pseudomonal keratitis is a potentially devastating ocular condition caused by the rapid ulceration of the cornea, which is a frequent occurrence. As in other forms of bacterial keratitis, the infection is characterized by an initial bacterial colonization of the corneal surface followed by invasion into deeper tissues, which then elicits an influx of inflammatory cells.1 The corneal destruction associated with Pseudomonas aeruginosa (PA) infection is thought to result from a complex mixture of proteases contributed by the bacteria, corneal cells, and inflammatory cells from the host.2-6

P. aeruginosa is known to produce at least two major matrix metalloproteinases (MMP), designated elastase and alkaline protease.7-10 Both have been reported to attack the helical structure of native type I collagen very slowly, whereas elastase has been reported to degrade native collagen types III and IV more rapidly, generating specific fragments.11 Both also have been reported to degrade rabbit corneal proteoglycans in vitro and in vivo.2,4,5 These enzymes have each been shown to play a role in the ulcerative process of Pseudomonas keratitis in rabbit models.2,12

Mammalian leukocytes synthesize and secrete several proteases. Human neutrophils secrete at least two MMPs and a serine protease: MMP-8 (neutrophil colla-
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genase), an enzyme that hydrolyzes collagen types I, II, and III;21 MMP-9 (92-kDa neutrophil gelatinase), an enzyme that hydrolyzes gelatin (partially denatured type I collagen) and collagen types IV and V;14,15; and neutrophil elastase, a serine protease that hydrolyzes collagen types III and IV, as well as the extracellular matrix constituents laminin, fibronectin, and heparin sulfate proteoglycan.16,17 Monocyte–macrophage cells produce serine proteases, including cathepsin G and leukocyte elastase, and several MMPs, including MMP-1 (interstitial collagenase), MMP-2 (72-kDa gelatinase), MMP-3 (stromelysin), MMP-7 (matrilysin or PUMP), and MMP-9 (92-kDa gelatinase).18-20

Corneal cells have been reported to produce matrix metalloproteinases. Gelatin zymography of detergent extracts of homogenized stroma from normal rabbit corneas revealed that corneal fibroblasts in vivo synthesize only the latent or potent form of MMP-2.21 Externs of the epithelium did not contain detectable gelatinase activity. In contrast, primary cultures of epithelial cells synthesized equivalent amounts of MMP-9 and small amounts of MMP-2, whereas cultures of stromal fibroblasts synthesized predominantly MMP-2 and smaller amounts of MMP-9.

The primary objective of current treatment strategies for Pseudomonas keratitis has been to sterilize the ulcer as rapidly as possible by using frequent topical dosing of an effective antibiotic. However, ulceration proceeds in some cases even when effective antibiotic treatment is administered. Ongoing corneal destruction may be caused by residual bacterial and inflammatory cell proteases. Thus, combining antibiotic treatment with protease inhibitors might improve clinical outcomes. Adjuvant therapies intended to reduce proteolytic activity have been limited generally to topical antiinflammatory agents or inhibitors of matrix metalloproteinases, such as tetracycline or N-acetyl cysteine. However, there are no studies to date that prove these inhibitors are effective clinically as an adjuvant therapy.

Newer inhibitors of matrix metalloproteinases have been developed that have higher potency and specificity than these previous compounds. These inhibitors contain chemical groups, such as hydroxamic acids or thiols, that interact with the zinc atom in the active site of MMP and amino acid groups complementary to the thiol of the substrate-binding domain of the active site of MMP. For example, the thiol-containing peptide HSCH2(DL)CH[CH2CH2(CH3)2]O-Phe-Ala-NH2 was found to be 10,000-fold more potent than N-acetyl cysteine and 1000-fold more potent than tetracycline when tested against collagenase purified from culture medium of alkali-burned rabbit corneas.22 This compound also was shown to inhibit Pseudomonas elastase with Ki = 300 nM and to delay the onset of corneal ulceration and perforation of rabbit corneas infected with Pseudomonas strain PA-28 in conditioned culture broth.23 Another thiol-containing compound, 2-mercaptoacetyl-L-phenylalanyl-L-leucine, inhibits Pseudomonas elastase with Ki = 200 nM and is reported to prevent melting of rabbit corneas infected with Pseudomonas elastase and to delay melting of corneas injected with washed Pseudomonas bacteria.24,25

Recently, the hydroxamic acid-containing modified dipeptide (N-[2(R)-2-(hydroxamido carbonyl)-4-methylpentanoyl]-L-tryptophane methylamide), also known as Galardin, was reported to block ulceration of alkali-burned rabbit corneas effectively.26 This compound inhibits purified Pseudomonas elastase with a Ki = 20 nM, inhibits MMP-1 (collagenase) with a Ki = 0.4 nM, and inhibits MMP-9 (gelatinase) with a Ki = 3 nM.26,27 Its activity against purified Pseudomonas alkaline protease has not been determined. In this study, we measured the ability of Galardin to inhibit protease activity in Pseudomonas culture broth in vitro, and we evaluated its effectiveness in reducing corneal destruction of rabbit corneas infected with Pseudomonas culture broth and corneas infected with washed Pseudomonas bacteria.

METHODS

Preparation of Pseudomonas Culture Broth

P. aeruginosa strain PA-28 was generously provided by Dr. Chris Patterson, University of Louisville, and strain W-186 was isolated from an ocular clinical specimen obtained at the University of Florida. Both strains were grown with vigorous shaking (220 rpm) at 37°C in Mueller–Hinton broth supplemented with calcium (1.25 mM) and magnesium (0.83 mM) to enhance protease production.12 Culture broth was filtered sterilized through a 0.22-μm pore filter and stored frozen at -20°C until used.

Analysis of Protease Activity in Pseudomonas Culture Broth

Gelatin zymography of culture broth was performed with some modifications of a previously described method.28 Briefly, samples of conditioned culture broth were incubated at room temperature with equal volumes of double-strength sample buffer containing sodium dodecyl sulfate (SDS) and without reducing agents (125 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 0.002% bromophenol blue) to permit recovery of protease activity after exchange of the SDS with Triton X-100. Samples were loaded in duplicate patterns on lanes of 8% polyacrylamide (wt/vol) gel in Tris buffer, pH 6.8, containing 0.1% gelatin. After
electrophoresis at 4°C, the gels were cut into two identical halves. One of the gel halves was washed twice for 30 minutes at 22°C in 2.5% Triton X-100 solution, then placed in 150 mM NaCl, 50 mM Tris, pH 7.8, 10 mM CaCl₂ for 18 hours at 37°C. The other half of the gel was washed with the same solutions except that Galardin was present at 8 µg/ml. The matrix metalloproteinase inhibitor Galardin was obtained from Glycomed (Alameda, CA). Galardin was formulated at a concentration of 800 µg/ml in vehicle consisting of 10 mM citrate, pH 5.5, 2.4% propylene glycol, and 0.05% methyl cellulose. Gels were then stained with Coomassie rapid stain (Diversified Biotech, Boston, MA) and photographed. Molecular weights of the protease bands were calculated by comparison of their migration distance relative to migration of protein standards with known molecular weights (broad-range prestained molecular weight standards; BioRad, Merville, NY).

General protease activities of the PA culture broths were measured using modifications of a previously described assay using Azocasein as the substrate. Briefly, 150 µl of culture broth or trypsin standard solution were added to 1.5 ml centrifuge tubes along with 100 µl of buffer (50 mM Tris buffer, pH 7.5) or 100 µl of inhibitor solutions. The reaction was initiated by the addition of 750 µl of Azocasein (Sigma Chemical, St. Louis, MO), which is a general protease substrate (4 mg/ml in 50 mM Tris buffer, pH 7.5). After 30 minutes of incubation at 37°C, 500 µl of 10% trichloracetic acid was added, the tubes were centrifuged at 10,000g for 4 minutes, and the absorbance of the supernatant solution was measured at 400 nm. Levels of protease activity were expressed as micrograms of trypsin per milliliter of culture broth. Dose–response curves for the serum protease inhibitor Aprotinin (1 mg/ml; Sigma) and the metalloproteinase inhibitor disodium ethylenediaminetetraacetic acid (EDTA) (0.5 M) were generated by adding serial dilutions of the inhibitors to the Azocasein reaction.

Rabbit Models of Pseudomonas Keratitis

This study conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research and was approved by the Animal Care and Use Committee of the University of Florida School of Medicine.

Noninfectious Keratitis Experiment. Injection of Pseudomonas Culture Broth A. Noninfectious rabbit model of Pseudomonas keratitis was used to determine initially the bioavailability and in vivo efficacy of Galardin treatment. Six female New Zealand white rabbits weighing 3 kg each were anesthetized for the duration of the experiment with intramuscular injections of ketamine and xylazine. To help mimic the epithelial defect that occurs during the course of Pseudomonas keratitis, an 8-mm vacuum trephine (Hessburg-Barron; Jedmed Instrument, St. Louis, MO) was placed centrally on the eye, n-heptanol was added to the well for 1 minute, and the well was aspirated and irrigated with 50 ml of phosphate-buffered saline (PBS). Heptanol exposure produced a sharply defined epithelial defect 8 mm in diameter to the central cornea, which stained with fluorescein. Epithelium surrounding the defect did not stain with fluorescein, and the conjunctiva appeared normal. One eye of each rabbit was then pretreated topically every 10 minutes for 1 hour with 2 drops of Galardin solution while the other eye was treated with vehicle to allow the inhibitor to penetrate the matrix before the proteases were injected. Each cornea was then injected intrastromally with 50 µl of filter-sterilized W-186 culture broth containing 38 µg of trypsin equivalent activity. Injections were made with the aid of an operating microscope using a 0.5-ml insulin syringe with a 30-gauge needle. Eyes were treated topically with 2 drops of vehicle or Galardin solution every 15 minutes for 6 hours. After 6 hours, 500 µl of Galardin or vehicle was injected subconjunctivally. Eyes were photographed and evaluated clinically at 0, 2.5, and 16 hours. Identities of the solutions were masked from the evaluator. Rabbits were killed after 16 hours, and the corneas were harvested for histologic examination.

Infectious Keratitis Experiment. Injection of Pseudomonas Bacteria Pseudomonas Strain. PA-28 was inoculated from a glycerol stock into 50 ml of Mueller–Hinton broth and grown overnight. One milliliter of the initial culture broth was added to 50 ml of fresh Mueller–Hinton broth and grown to an optical density of 0.40 at 600 nm (Spectronic 20; Bausch & Lomb, Rochester, NY), which corresponded to a concentration of 1.38 × 10⁶ bacteria per milliliter (determined from a standard curve of absorbance versus colonies counted on LB agar plates). Twenty milliliters of the PA-28 culture was centrifuged at 10,000g for 10 minutes, resuspended in 20 ml of PBS, centrifuged again, and resuspended in 2.5 ml of PBS to give a final concentration of 1.38 × 10⁷ bacteria per milliliter (confirmed by counting colonies on LB agar plates of serial dilutions of the injection suspension).

Four female New Zealand white rabbits weighing 2.5 kg each were anesthetized as described above. Using a stereoscopic operating microscope, a 4 × 4 mm central area of epithelium was removed using a #15 scalpel blade. Twenty microliters of the bacterial solution containing 2.76 × 10⁷ bacteria were injected into the central corneal stroma using a 50-µl Hamilton syringe with a 30-gauge needle. Injections of butorphenol tartrate were given for analgesia.

Beginning 15 minutes after injection, each rabbit
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received 2 drops of Galardin in one eye and 2 drops of vehicle in the other eye every 15 minutes throughout the 26-hour study. Treatments were administered in a masked fashion, and corneal pathology was assessed grossly and with a slit lamp biomicroscope. Clinical scores were recorded every 2 hours by the same examiner using the following scale: 1 = injection edema and injury, no other pathology; 2 = melting or abscess extending over area < \( \frac{1}{3} \) corneal diameter; 3 = melting or abscess extending over area > \( \frac{1}{3} \) and < \( \frac{2}{3} \) corneal diameter and/or mild bulging; 4 = melting or abscess extending over area > \( \frac{2}{3} \) corneal diameter and/or moderate bulging; 5 = severe bulging or descemetocele. Clinical scores were compared for statistical differences using a one-way repeated measure analysis of variance, which assumes a constant variability of the data over time and an underlying normal distribution of the data.

RESULTS

Gelatin Zymography of Pseudomonas Culture Broth

Culture broth from W-186 strain contained two proteases that hydrolyzed gelatin (Fig. 1). Molecular weights calculated from their migration distances were 123 kDa and 55 kDa. Gelatinase activities of both bands were blocked by the addition of Galardin during the exchange of SDS and incubation at 37°C. Gelatin zymography of culture broth from PA-28 also generated two bands with the same molecular weights that were inhibited by Galardin.

Effect of Inhibitors on Protease Activity of Pseudomonas Culture Broth

Filter-sterilized culture broth from the W-186 strain of PA contained substantial protease activity (763 \( \mu \)g trypsin equivalent/ml) using Azocasein as the substrate. Addition of Galardin to the culture broth (final concentration of 80 \( \mu \)g/ml in the assay) reduced the level of proteolytic activity by 99% to 7 \( \mu \)g/ml. In a separate assay of W-186 broth, serial dilutions of Galardin also reduced protease activity by 99% at a final inhibitor concentration of 8 \( \mu \)g/ml, reduced activity by 68% at 0.8 \( \mu \)g/ml, and reduced activity by 46% at 0.08 \( \mu \)g/ml, demonstrating a dose-dependent inhibition. The vehicle alone (10 mM citrate, pH 5.5, 2.4% propylene glycol and 0.05% methyl cellulose) did not affect protease activity of the culture broth.

Similar trends were found with inhibitor treatment of culture broth from strain PA-28. Addition of Galardin at a final concentration of 80 \( \mu \)g/ml reduced protease activity of PA-28 culture broth by 99%. Addition of 50 mM EDTA also reduced PA-28 protease activity by 99%. Addition of the serine protease inhibitor, aprotinin, at 100 \( \mu \)g/ml did not significantly reduce PA-28 proteolytic activity. Thus, nearly all the Azocasein-degrading proteases secreted by P. aeruginosa in Mueller–Hinton broth appeared to be matrix metalloproteinases because Galardin and EDTA were both effective inhibitors.

Effect of Galardin in the Noninfectious Model of Pseudomonas Keratitis

Injection of 50 \( \mu \)l of sterile culture broth immediately produced a central stromal opacity approximately 8 mm in diameter that spontaneously resolved after approximately 1 hour. After 2.5 hours, all six eyes treated with vehicle began displaying liquefaction and opacification (Fig. 2, panel A). In contrast, eyes treated with Galardin were clear and showed no observable destruction other than the initial epithelial defect (Fig. 2, panel B). After 16 hours, vehicle-treated eyes were near perforation (Fig. 2, panel C), whereas the inhibitor-treated eyes were clear and lacked detectable inflammatory cell infiltrate (Fig. 2, panel D). Histo-
broth. Otherwise, the middle and posterior stroma had normal arrangement of the collagen lamellae (Fig. 2, panel F).

**Effect of Galardin in the Infectious Model of Pseudomonas Keratitis**

Injection of 20 μl of PBS containing $2.76 \times 10^7$ washed PA-28 bacteria immediately produced a central opacity in the corneal stroma that spontaneously resolved to a slight haze within 1 hour. Corneas treated with vehicle began to show significant melting at an average of 12 hours from the time of inoculation (Fig. 3). In contrast, corneas treated with Galardin averaged more than 22 hours before the initiation of melting (one cornea did not begin melting by the end of the experiment at 26 hours). Thus, Galardin treatment delayed initiation of ulceration an average of 10 hours compared to vehicle-treated eyes. Although treatment with Galardin substantially delayed the initiation of corneal ulceration, it did not prevent eventual progression of corneal destruction. The maximum difference in average clinical scores for the two treatment arms occurred at 22 hours (2.25 units) and decreased to 1.75 units at 26 hours. Figure 4 shows the appearance of vehicle-treated (panel A) and Galardin-treated (panel B) eyes at 20 hours, near the point at which there was
maximal difference in the extent of corneal melting between the two treatment groups.

Statistical analysis of the average clinical scores indicated there was a significant difference in mean clinical scores between corneas treated with Galardin and those treated with vehicle at all evaluation time points between 16 and 26 hours. Probability values at each time point ranged from $P = 0.005$ at 16 hours to $P = 0.0001$ at 22 hours. For each of the four rabbits, the clinical score of the vehicle-treated eye was equal to or greater than the clinical score of the Galardin-treated companion eye at all time points throughout the experiment until 26 hours, when one Galardin-treated eye reached a clinical score of 5 and had a vehicle-treated companion eye with a score of 4.

DISCUSSION

Among the various pathogens encountered in bacterial keratitis, *P. aeruginosa* causes the most devastating injury. Furthermore, it is the most common pathogen causing corneal ulcers in contact lens wearers. Pseudomonal corneal ulcers can evolve rapidly, even when timely and appropriate antibiotic treatment is administered. This may be because of the continued release of inflammatory enzymes and the presence of residual bacterial proteases in the cornea even after the bacteria have been killed by topical antibiotic therapy.

Because the majority of bacterial enzymes and many of the host-derived enzymes are metalloproteinases, various metalloproteinase inhibitors have been evaluated as potential adjunctive treatments. Matrix metalloproteinase inhibitors such as EDTA, N-acetylcysteine (Mucomyst), and tetracycline initially were evaluated in animal models of corneal alkali injuries and were found to reduce ulceration partially. Systemic tetracycline was reported to reduce the incidence of corneal perforation in a rabbit model of *Pseudomonas* keratitis. Newer-generation sulphydryl-containing compounds (2-mercaptoacyetyl-L-phenylalanyl-L-leucine and HSCH$_2$CH[CH$_2$CH(CH$_3$)$_2$]CO-Phe-Ala-NH$_2$), which are more potent MMP inhibitors than tetracycline or N-acetylcysteine, have demonstrated efficacy in reducing corneal ulceration in rabbit models of *Pseudomonas* keratitis. Recently, the hydroxamic acid-containing modified dipeptide inhibitor Galardin was developed. It has approximately 10- to 15-fold higher potency against *Pseudomonas* elastase than the sulphydryl inhibitors.

Results from the gelatin zymograms demonstrated that culture broth from W-186 and PA-28 strains contained two major gelatinases with molecular weights of approximately 123 kDa and 55 kDa, both inhibited by Galardin. Molecular weights of these two proteases agree closely with those reported for purified *Pseudomonas* elastase and alkaline protease, respectively, in nonreducing gelatin zymography. These differ from the molecular weights predicted from the gene sequences for elastase of 34 kDa and for alkaline protease of 49.5 kDa as well as the molecular weights of 30 to 35 kDa and 50 to 55 kDa reported for the purified proteases analyzed in reduced SDS polyacrylamide gels. These differences, in apparent molecular weights, probably result from the formation of aggregates of the proteases that are stable to the mild conditions of sample equilibration used for the zymography (22°C and no reducing agents versus boiling the samples for 5 minutes with reducing agents used for denaturing SDS gel electrophoresis). It is unknown whether the proteases aggregate with themselves or with other proteins. Recently, a new metalloproteinase that migrated with an apparent molecular weight of 97 kDa in gelatin zymography was detected in *Pseudomonas* strain 115. This protease was not detected in culture broth from W-186 or PA-28 strains.

Protease activities in culture broth from W-186 or PA-28 strains were analyzed quantitatively using...
Azocasein as the substrate. Galardin inhibited nearly all proteolytic activity in both culture broths, whereas the serine protease inhibitor aprotinin was ineffective. This indicates that a majority of the proteolytic activity in *Pseudomonas* culture broth is of the metalloproteinase class, which is consistent with previous reports indicating that the major proteases produced by *Pseudomonas* strains are elastase and alkaline protease.7-10

Results of the noninfectious in vivo model show that Galardin prevented acute corneal destruction caused by products present in *Pseudomonas* culture broth. Because Galardin is an inhibitor of metalloproteinases, it suggests that matrix metalloproteinases are the principle bacterial products responsible for the acute corneal destruction observed in this animal model. This is consistent with the results of Twining and colleagues,23 who reported that the level of proteolytic activity in culture broth of various strains of PA correlated with the production of descemetoceles in two rabbit PA keratitis models. However, previous studies conducted with mutant strains of PA bacteria indicated that pathogenesis of PA keratitis is multifactorial. Exotoxin A, an adenosine diphosphate-ribosyl transferase produced by PA that inhibits protein synthesis by blocking the action of elongation factor 2, is important for virulence and was reported to play a role in corneal tissue destruction in mouse corneas.98 Because protein synthesis probably is not a crucial factor in this model of acute corneal destruction, the effects of exotoxin A may not be detected readily.

Results of the infectious model *Pseudomonas* keratitis demonstrate that Galardin significantly delayed, but did not prevent, eventual corneal destruction after the injection of washed, live PA. Burns and colleagues26 also were able to delay, but not prevent, eventual corneal destruction by topical treatment with a sulfhydryl-based MMP inhibitor after the injection of PA suspended in culture broth conditioned by 18 hours of bacterial growth. The onset of corneal destruction observed in their control group occurred substantially earlier than in our experiment (3 hours versus 12 hours), probably because proteases already were present in the conditioned culture broth. However, it is unknown whether PA synthesizes the same types and levels of proteins in the cornea that it does in conditioned culture broth. Recently, enzymatically active alkaline protease, but not elastase, was detected in corneal tissue of mice with experimental *Pseudomonas* keratitis.86 Thus, injection of washed PA bacteria may parallel clinical infections more closely than injection of PA suspended in conditioned culture broth.

There may be several factors that contribute to the inability of Galardin to prevent eventual corneal destruction in this model of *Pseudomonas* keratitis. Probably the most important factor is that serine proteases from inflammatory cells not inhibited by Galardin, such as neutrophil elastase, may increase to a level that is sufficient to degrade the corneal matrix. If this explanation is correct, then a combination of inhibitors against MMP and serine proteases may prove to be more effective than treatment with a single class of protease inhibitor.

Another possibility is that the concentration of MMP contributed by both the *Pseudomonas* bacteria and by inflammatory cells reaches such a high level that even inhibition of a very high percent (99%) of MMP molecules leaves a significant amount of MMP activity, which eventually degrades the stromal matrix. Results of unpublished experiments we conducted to assess the uptake of Galardin into rabbit corneas determined that 6 hours after initiating hourly eye drops of Galardin to rabbits, the concentration of Galardin in the corneal stroma was 150 nM. Even though the rabbits injected with PA were treated more frequently with Galardin (every 15 minutes throughout the experiment) and the Ks of Galardin for *Pseudomonas* elastase was low (20 nM), approximately 1% of the total MMP activity in the cornea would have remained active even if the concentration of Galardin in the cornea reached 100X the Ks (or 2000 nM). Thus, if the amount of *Pseudomonas* protease activity in the cornea is large, inhibition of 99% of the protease molecules would still leave a substantial amount of protease activity, which could contribute to corneal destruction.

In summary, these results indicate that treatment of experimental *Pseudomonas* keratitis with the metalloproteinase inhibitor, Galardin, significantly reduced the rate of corneal ulceration by inhibiting bacterial and inflammatory cell MMP. Recently, a clinical trial of Galardin as an adjuvant to antibiotics for the treatment of infected corneal ulcers was completed. According to manufacturer claims, Galardin treatment resulted in a statistically significant reduction in the incidence of corneal perforations as well as in the proportion of patients whose ulcers became very deep. This suggests that Galardin, when used as an adjuvant to antibiotics, reduces the incidence of severe complications of infectious corneal ulcers. These results justify future investigations of protease inhibitors as a treatment for ulcerative conditions of the anterior segment—such as corneal alkali burns, necrotizing scleritis, and posterior keratitis—in which bacterial and inflammatory cell proteases are thought to play a prominent role.

**Key Words**
corneal ulceration, metalloproteinases, protease inhibitors, *Pseudomonas* keratitis

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