Inhibition of *Pseudomonas aeruginosa* Elastase and *Pseudomonas* Keratitis Using a Thiol-Based Peptide

FRANK R. BURNS,1,* CHRISTOPHER A. PATERSON,1 ROBERT D. GRAY,1,2 AND JOHN T. WELLS1

Department of Ophthalmology and Visual Sciences1 and Department of Biochemistry,2
University of Louisville School of Medicine, Louisville, Kentucky 40292

Received 2 April 1990/Accepted 16 August 1990

*Pseudomonas aeruginosa* elastase is a zinc metalloproteinase which is released during *P. aeruginosa* infections. *Pseudomonas* keratitis, which occurs following contact lens-induced corneal trauma, can lead to rapid, liquefactive necrosis of the cornea. This destruction has been attributed to the release of both host-derived enzymes and the bacterial products *P. aeruginosa* elastase, alkaline protease, exotoxin A, and lipopolysaccharide endotoxin. A synthetic metalloproteinase inhibitor, HSCH2(CH2)6CH2CH2Cl-Phe-Ala-NH2, which we previously showed to be a potent inhibitor of corneal collagenase and alkali-induced corneal ulceration, was tested as a potential inhibitor of *P. aeruginosa* elastase. Inhibition constants (Ki) for the resolved diastereomers were determined with the chromogenic substrate furylacryloyl-glycyl-l-leucyl-l-alanine. One isomer had a Ki of 0.3 μM, while the other had a Ki of 0.4 μM. The more potent diastereomer was evaluated in vivo in experimentally induced *Pseudomonas* keratitis in rabbits. Following inoculation of one cornea of each rabbit, topical treatment with a 1 mM solution of the inhibitor significantly delayed the onset of corneal melting and perforation, as compared with the results for the control and gentamicin-treated groups. This protective effect suggests that the inhibitor may have a therapeutic application by delaying the progression of corneal destruction in *Pseudomonas* keratitis.

*Pseudomonas aeruginosa* is one of the most commonly encountered organisms found in infectious keratitis (26, 28). *Pseudomonas* keratitis causes rapid, liquefactive destruction of the human cornea and is being seen with increasing frequency following corneal trauma from extended-wear contact lenses (7, 12). Destruction of the cornea in *Pseudomonas* keratitis results from the release of degradative enzymes from both the organism (3, 16, 18, 24) and host-derived sources (17). The metalloproteinase *P. aeruginosa* elastase is one of the main enzymes released by *P. aeruginosa* and is thought to be a major contributor to the pathogenesis of the organism (16, 24).

It has been demonstrated that immunization of rabbits and mice with elastase provides protection from *Pseudomonas* keratitis (25). Recent studies have suggested that inhibition of *P. aeruginosa* elastase may lead to more effective therapeutic approaches to this disease (19, 21, 22). Compounds which inhibit metalloproteinases such as *P. aeruginosa* elastase have been shown to alter the clinical course of *Pseudomonas* keratitis. The compound 2-mercaptoacetyl-l-Phe-l-Leu, an inhibitor of *P. aeruginosa* elastase (17), reduced the amount of corneal melting in experimental *Pseudomonas* keratitis in rabbits when combined with gentamicin (31). Recently, tetracycline was shown to reduce the incidence of alkali-induced corneal ulceration (30) and *Pseudomonas* keratitis-induced corneal perforation in rabbits (27). This effect was thought to be secondary to the anticollogenase effect of tetracycline.

The metalloproteinase inhibitor HSCH2(CH2)6CH2CH2Cl-Phe-Ala-NH2 is a potent inhibitor of mammalian collagenases, such as pig synovial collagenase (8) and purified collagenase from alkali-burned rabbit corneas (6). It also inhibits alkali-induced corneal ulceration and perforation in rabbits (4). In the present study, we found that the peptide is a potent inhibitor of *P. aeruginosa* elastase in vitro. Because the peptide inhibited both corneal collagenase and elastase, we postulated that the inhibitor would be more effective against *Pseudomonas* keratitis than are compounds which inhibit only elastase (31). Our findings indicate that the inhibitor slowed the progression of *Pseudomonas* keratitis and was more effective than was either vehicle or topical gentamicin in this model.

MATERIALS AND METHODS

**Bacterial strain and growth conditions.** A virulent strain of *P. aeruginosa*, PA-28 (donated by P. V. Liu, Department of Microbiology and Immunology, University of Louisville School of Medicine), was used throughout this study. Stock cultures were kept frozen in Trypticase soy broth with 20% glycerol at −20°C. For enzyme production, bacteria were incubated with shaking at 37°C for 48 h in Mueller-Hinton broth (Difco Laboratories).

**Elastase purification.** *P. aeruginosa* elastase was purified from the bacterial filtrate by ammonium sulfate precipitation (60% saturation) and molecular sieve chromatography with a Bio-Gel A0.5m column as previously described (18). An azocasein assay was used to separate elastase from alkaline protease (17). Solutions of the purified enzyme (0.4 to 1.2 mg/ml) in 0.01 M Tris hydrochloride (pH 7.45) were stored in aliquots at −20°C.

**Inhibitors.** The thiol inhibitors were synthesized and characterized as described previously (8, 11). The diastereomers were resolved by C18 reversed-phase high-pressure liquid chromatography. The inhibitors were dissolved in 95% ethanol containing 1 mM acetic acid immediately prior to use, and the thiol titers were determined by the Ellman procedure (9).

**Enzyme assays.** The chromogenic substrate furylacryloyl-glycyl-l-leucyl-l-alanine (FA-Gly-Leu-Ala) was prepared as previously described (2). Peptidase activity was assayed with FA-Gly-Leu-Ala at 25°C with a Varian-Cary 219 spec-
trophotometer interfaced to a microcomputer (On-Line System, Jefferson, Ga.) by monitoring the decrease in the $A_{540}$ caused by the hydrolysis of the Gly-Leu bond (2, 10). All reactions were carried out at 25°C in 0.1 M NaCl-0.05 M Tris hydrochloride-0.01 M CaCl$_2$ (pH 7.5) and at FA-Gly-Leu-Ala concentrations of 0.1 to 0.2 mM. Inhibition constants ($K_i$s) were derived from plots of $k_{obs}/k$ versus inhibitor concentration ($k_i$, and $k$ are the observed rate constants in the absence and in the presence of the inhibitors, respectively) (17).

Inhibitor and antibiotic preparation for in vivo studies. The inhibitor used for topical and subconjunctival treatment was prepared in the same manner as that described previously (4), with the exception that the inhibitor was dissolved in dimethyl sulfoxide instead of ethyl alcohol and acetic acid. The final inhibitor concentration was 1 mM. Vehicle was also prepared as previously described, with the exception that dimethyl sulfoxide was used instead of ethyl alcohol and acetic acid (4).

Gentamicin sulfate was prepared by the pharmacy at Humana Hospital, University of Louisville, by adding injectable gentamicin solution to balanced salt solution to a final concentration of 15 mg/ml. The solution was made on the day of the experiment and was kept at 4°C when not being used. Balanced salt solution without preservatives was used as the control vehicle.

Bacterial preparation for in vivo studies. *P. aeruginosa* PA-28 is susceptible to gentamicin in vitro (MIC, 2 μg/ml). Stock cultures of the organism were kept in Trypticase soy broth with 20% glycerol at −20°C. For experiments, a suspension of organisms from the stock was grown overnight on Mueller-Hinton agar. Direct inoculations were then made from the overnight agar growth into Mueller-Hinton broth (20 ml) in 250-ml shaker flasks. Extra Ca$^{2+}$ (50 μg/ml) and Mg$^{2+}$ (20 μg/ml) were added to the broth to increase protease production (32). The suspension was incubated with shaking at 37°C for 16 to 18 h. At the end of the incubation period, the suspension was diluted to an $A_{540}$ of 0.2 with Mueller-Hinton broth. This value corresponded to a final concentration of 6.0 × 10$^8$ cells per ml.

Corneal inoculations and treatment regimens. Forty-eight New Zealand White rabbits weighing between 2 and 2.5 kg were used. The methods used in this study were in accord with the Association for Research in Vision and Ophthalmology Resolution for the Use of Animals in Research. The animals were anesthetized by intramuscular injection of ketamine hydrochloride (37.5 mg/kg) and xylazine hydrochloride (5 mg/kg) and by topical application of tetracaine. Following anesthetization, one eye of each animal was subjected to propnosis, and a central area of corneal epithelium approximately 4 mm in diameter was removed by scraping with a scalpel blade. Twenty microliters of the *P. aeruginosa* cell suspension (1.2 × 10$^8$ cells) was injected intrastromally into the center of the cornea with the use of a 30-gauge needle and a 100-μl Hamilton syringe. All injections were performed with the aid of a stereoscopic microscope. Animals were given buprenorphine hydrochloride (0.05 mg/kg) intramuscularly as needed for pain control.

In three independent experiments, the rabbits were divided randomly into either a treatment group or a control group (six eyes per group in experiment A and nine eyes per group in experiments B and C). Experiment A included a group treated with inhibitor both topically and subconjunctivally according to the treatment schedule in Table 1. In experiment A, topical treatment was begun 2 h following bacterial inoculation. The control group received vehicle only. Treatment was continued until the end of the study, 26 h postinoculation. Experiment B included a group treated with topical inhibitor alone according to a more frequent dosing schedule than that used in experiment A (Table 1). In experiments B and C, topical treatment was begun immediately following bacterial inoculation because of the rapid progression of corneal melting and perforation noted in experiment A. Treatment was continued to the end of the studies, 24 h postinoculation. A control group was also included. Experiment C compared treatment with topical fortified gentamicin drops to treatment with vehicle alone (Table 1). One of us (F.R.B.) examined the rabbits in a masked fashion every 2 h in experiment A. This schedule was changed to every hour for experiments B and C because of the rapid progression of corneal melting and perforation noted in experiment A. Twelve hours following inoculation, examinations were made every 2 h through 24 h. The severity of the corneal pathology was estimated grossly and scored according to the criteria shown in Table 2. The clinical scoring criteria were a modification of criteria used in previous studies (4, 31).

### RESULTS

#### Inhibition of *P. aeruginosa* elastase by a synthetic metalloproteinase inhibitor. Inhibition constants ($K_i$s) were derived

<table>
<thead>
<tr>
<th>Exp</th>
<th>Route</th>
<th>Dosage</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Topical$^a$</td>
<td>1 mM inhibitor every 15 min for 2 h and then every hour for 10 h</td>
</tr>
<tr>
<td></td>
<td>Subconjunctival$^b$</td>
<td>1 mM inhibitor in 0.5 ml of saline at 10 h postinoculation</td>
</tr>
<tr>
<td></td>
<td>Topical$^a$</td>
<td>1 mM inhibitor every hour from 18 to 26 h postinoculation</td>
</tr>
<tr>
<td>B</td>
<td>Topical$^a$</td>
<td>1 mM inhibitor every 15 min for 4 h and then every 30 min for 20 h postinoculation</td>
</tr>
<tr>
<td>C</td>
<td>Topical$^a$</td>
<td>Fortified gentamicin every 15 min for 4 h and then every 30 min for 20 h postinoculation</td>
</tr>
</tbody>
</table>

$^a$ The control group received vehicle topically on the same schedule.  
$^b$ The control group received saline subconjunctivally.

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### TABLE 2. Clinical scoring criteria for experimental *Pseudomonas* keratitis

<table>
<thead>
<tr>
<th>Score</th>
<th>Clinical finding</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>No corneal pathology</td>
</tr>
<tr>
<td>1</td>
<td>Corneal infiltrate without significant melting</td>
</tr>
<tr>
<td>2</td>
<td>Grade I melting (none to one-third of the corneal surface)</td>
</tr>
<tr>
<td>3</td>
<td>Grade II melting (one-third to two-thirds of the corneal surface) and/or mild bulging</td>
</tr>
<tr>
<td>4</td>
<td>Grade III melting (two-thirds to all of the corneal surface) and/or moderate bulging</td>
</tr>
<tr>
<td>5</td>
<td>Descemetocoele formation and/or severe bulging</td>
</tr>
<tr>
<td>6</td>
<td>Corneal perforation</td>
</tr>
</tbody>
</table>
for the metalloproteinase inhibitor and its stereoisomer by measuring the inhibition of the enzymatic hydrolysis of the synthetic substrate FA-Gly-Leu-Ala. The more slowly eluting isomer of the synthetic inhibitor had a $K_i$ of 0.3 $\mu$M, while the more rapidly eluting isomer had a $K_i$ of 0.4 $\mu$M.

**Inhibition of Pseudomonas keratitis.** The average clinical scores in experiments A, B, and C are shown graphically in Fig. 1, 2, and 3, respectively. Figure 1 demonstrates that the inhibitor alone when used topically and subconjunctivally significantly reduced the clinical scores for the corneas in comparison with those for the control corneas. Clinical scores were significantly lower from 6 through 12 h ($P < 0.05$) following inoculation. At 10 h following inoculation of the corneas with *P. aeruginosa*, five of the six control eyes had perforated corneas, whereas none of the inhibitor-treated eyes had perforated corneas.

The results of experiment A indicated that topical treatment with the inhibitor could have a protective effect on *P. aeruginosa*-infected corneas. This effect was found to last through 8 h postinoculation, when stromal melting of the inhibitor-treated corneas was first noted. Because corneal melting of the eyes treated with the inhibitor progressed more rapidly when topical treatment was interrupted, a second experiment (B) was designed to test whether increasing the frequency and duration of topical treatment would have a greater protective effect. Topical treatment was started immediately following inoculation, with treatments given every 15 min for the first 4 h and then every 30 min for the next 20 h. The results are shown in Fig. 2. Corneal melting of the inhibitor-treated eyes progressed more slowly than did that of the vehicle-treated eyes. The first perforation in the control group was noted at 3 h postinoculation, whereas the first perforation in the inhibitor-treated group was not noted until 14 h postinoculation. The mean clinical score for the inhibitor-treated group was significantly lower than that for the control group from 3 h ($P < 0.02$) through 18 h ($P < 0.05$) postinoculation ($P < 0.001$ from 5 through 12 h postinoculation). Thus, the peptide significantly slowed the onset of corneal melting and perforation. No corneal melting was noted in the inhibitor-treated eyes until 12 h postinoculation, at which time five of the nine control eyes already had perforated corneas.

A third experiment (C) was performed to evaluate the response of this model to antibiotic therapy. After establishing that the *P. aeruginosa* strain used was susceptible to gentamicin (MIC, 2 $\mu$g/ml), we treated one group of rabbits with fortified gentamicin solution and a second group with...
vehicle only (balanced salt solution) according to the same treatment schedule as that used in experiment B. The results are shown in Fig. 3. Treatment with fortified gentamycin had no protective effect against corneal destruction. There was no significant difference between the mean clinical scores for the gentamycin-treated and vehicle-treated eyes at any time following inoculation of the corneas. At 12 h postinoculation, six of nine gentamycin-treated corneas were perforated, as were six of nine vehicle-treated corneas.

**DISCUSSION**

*P. aeruginosa* has become a commonly isolated pathogen in bacterial keratitis (26, 28). The rise in its occurrence has been attributed to the increased use of extended-wear contact lenses (7, 12). Following adherence of the organism to a damaged corneal epithelium (14, 29), the release of exogenous enzymes, including proteases (16), elastases (16, 24), and exotoxins (1, 13, 15), is necessary for stromal penetration of the organism to occur. Evidence suggests that these enzymes contribute to corneal destruction (3, 16, 18, 24) and that their inhibition may lead to a reduction in the amount of corneal destruction which occurs (17, 22, 23).

The extracellular protease *P. aeruginosa* elastase is thought to contribute to the rapid liquefactive dissolution of the cornea during *Pseudomonas* keratitis (16, 24). Spierer and Kessler (31) demonstrated that the compound 2-mercaptoacetyl-L-Phe-L-Leu, an inhibitor of *P. aeruginosa* elastase (17), had a protective effect against *Pseudomonas* keratitis when combined with gentamycin. The effect was significant at 28 h but not at 48 h postinoculation (31). A recent study (27) also showed that systemic ticrynafine treatment reduced corneal perforation due to *Pseudomonas* keratitis in rabbits from 80 to 45% over a period of 10 days. This effect was independent of any antimicrobial effect and was hypothesized to be secondary to the anticollegenase activity of ticrynafine (27). The recent finding by our laboratory that ticrynafine compounds inhibit *P. aeruginosa* elastase suggests that the reduction in corneal pathology may be due to this action instead of or in addition to the anticollegenase activity of ticrynafine (5).

In addition to the enzymes released by the organism, host-derived sources of degradative enzymes appear to play a role in the destructive nature of *Pseudomonas* keratitis. A previous study demonstrated that when heat-killed *P. aeruginosa* cells were injected intrastromally into the cornea, corneal melting and ulceration progressed in a manner similar to that seen with live *P. aeruginosa* (19). Analysis of the corneal tissue revealed the presence of collagenolytic activity in the ulcerating corneas, and histologic examination revealed an influx of polymorphonuclear leukocytes into the corneal stroma. Thus, it was hypothesized that host-derived collagenase and other proteases may play a significant role in the rapid corneal melting seen in *Pseudomonas* keratitis.

We have shown in the present study that the synthetic metalloproteinase inhibitor HSCH₂(α,CH₂CH₂CH₃)CO-Phe-Ala-NH₂ inhibits *P. aeruginosa* elastase. We showed previously that it is a powerful inhibitor of corneal and synovial collagenases (6, 8) and also inhibits alkali-induced corneal ulceration and perforation in rabbits (4). Because our compound inhibited both *P. aeruginosa* elastase and corneal collagenase, we felt that it may provide more corneal protection against *Pseudomonas* keratitis than does the compound evaluated by Spierer and Kessler (31). The large inoculum used in the present study (1.2 × 10⁷ organisms) was chosen to evaluate the response of the inhibitor to rapidly destructive keratitis, similar to the model used by Spierer and Kessler (31). Treatments were begun at time zero in experiments B and C because of the large inoculum and the rapid progression of corneal melting noted in experiment A.

Our results indicate that the inhibitor delayed the onset of corneal melting and perforation in this model of *Pseudomonas* keratitis. We also found that increasing the frequency of topical therapy resulted in a longer delay in the onset of corneal melting and perforation. Finally, we showed that therapy with topical fortified gentamycin had no effect upon corneal destruction in this model. This finding was of interest because the organism was highly susceptible to gentamycin in vitro (MIC, 2 μg/ml). The most likely explanation for this is that the gentamycin could not kill such a large inoculum of organisms (1.2 × 10⁷). Another explanation may be that the broth had such a high concentration of enzymes when injected into the cornea that the inhibition of bacterial growth could not prevent the rapid corneal destruction which occurred. The inhibitor most likely had no effect upon bacterial growth within the cornea, as Kessler and Safrin have shown that thiol-based protease inhibitors do not affect the growth of *P. aeruginosa* in cultures (20).

The inhibitory effect of the peptide did not last through 24 h. One explanation may be that other enzymes which were not inhibited by the peptide were released by the organism. Polymorphonuclear leukocytes, which readily infiltrate the cornea in bacterial keratitis, could have also released degradative enzymes, such as polymorphonuclear leukocyte elastase, which were not inhibited by the peptide. The organism may have inactivated the inhibitor, as is the case with other thiol-based peptides (21).

It is well recognized that even with optimum antibiotic therapy begun as soon as a corneal ulcer is detected, the cornea often progresses rapidly to melting, ulceration and, frequently, perforation (26, 28). In the case of *Pseudomonas* keratitis, progression to perforation can occur as early as 2 to 5 days following initial presentation. In these cases, it seems likely that additional therapeutic measures which are aimed at inhibiting the enzymes produced by the organism and by the host would have a beneficial effect when combined with an appropriate antibiotic regimen. We believe that the present study demonstrates that our synthetic metalloproteinase inhibitor may aid in the prevention of corneal melting and perforation in *Pseudomonas* keratitis.

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**LITERATURE CITED**


