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Inhibition of Purified Collagenase from Alkali-Burned Rabbit Corneas

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The inhibitory potency of four classes of compounds that inhibit corneal ulceration (thiols, tetracyclines, sodium citrate and sodium ascorbate) was assessed with collagenase purified from culture medium of alkali-burned rabbit corneas. The most potent inhibitor, a β-mercaptomethyl tripeptide $HSCH_2(DL)CH[CH_2CH(CH_3)_2]CO$-Phe-Ala-$NH_2$, exhibited 50% inhibition (IC_{50}) at ~10 nM using the synthetic metalloproteinase substrate Dnp-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2. The inhibitor was somewhat less potent with type I collagen as substrate (IC_{50} between 1 and 3 μM), possibly because autooxidation of the essential −SH moiety of the inhibitor occurred during the longer time required for assay with the natural substrate. An N-carboxyalkyl tripeptide, $CH_3(CH_2)_2(DL)CH$-(COOH)-Leu-Phe-Ala-NH_2, was less potent (IC_{50} = 25 μM) than the thiol peptide. N-acetylcysteine, which is used to treat corneal ulceration, gave IC_{50} values of 2.7 mM and >10 mM with the synthetic and natural substrates, respectively. The IC_{50} values for the tetracyclines using the synthetic substrate were 15, 190 and 350 μM for doxycycline, minocycline and tetracycline, respectively. Inhibition by sodium citrate, but not the tetracyclines, could be reversed by excess Ca^{2+}. Sodium ascorbate did not inhibit collagenase-mediated hydrolysis of either collagen or the synthetic substrate, thus indicating that the mechanism by which this agent inhibits corneal ulceration is not related to inhibition of collagen degradation by collagenase. Invest Ophthalmol Vis Sci 30:1569–1575, 1989

Treatment of the alkali-burned eye continues to be a major challenge to the ophthalmologist.[1] Many therapeutic techniques have been used in an attempt to prevent the sequellae which threaten the integrity of the eye following a chemical injury. These include corticosteroids, heparin, collagenase inhibitors, contact lenses, fibronectin, conjunctival flaps and corneal transplantation.[1,2] Recent studies have advocated the use of sodium citrate and sodium ascorbate.[3-5] Following an ocular alkali burn, a number of degradative processes occur which may result in a corneal ulcer. Several proteases, including collagenase,[6-8] are elaborated in the chemically injured cornea and account for the ulcerative process. Although the multitude of treatment modalities used in these injuries undoubtedly work by different mechanisms of action, successful management of ocular alkali burns requires the use of agents which reduce the impact of collagenase and other proteases upon the cornea.[1] Therefore, a well justified approach and direction of research has been to test inhibitors of collagenase and other host-derived proteases in the treatment of the alkali-burned eye.

The efficacy of inhibitors of collagenase for use in human corneal alkali burns is open to question.[1] Compounds that have been tested experimentally in animals include acetylcysteine,[9] cysteine,[9,10] sodium and calcium EDTA[11] and penicillamine[12]; of these, acetylcysteine (Mucomyst®, Bristol–Myers, New York, NY), which is approved for use as a mucolytic agent, is the only collagenase inhibitor used clinically in the treatment of human alkali burns.[1,2] Its efficacy has yet to be proven in a randomized clinical trial.[2] Collagenase inhibition by the tetracycline family of antibiotics has been demonstrated in vitro[13-16] and systemic tetracycline has recently been shown to inhibit alkali-induced corneal ulceration in rabbits.[17]

Synthetic peptides that are highly potent inhibitors of mammalian sources of collagenase have been recently developed by Gray et al.[18-20] The purpose of the current study was to evaluate the inhibition of purified corneal collagenase by two of these synthetic peptides in comparison with compounds currently known to inhibit collagenase. Qualitative indications of inhibitor potencies were first determined using

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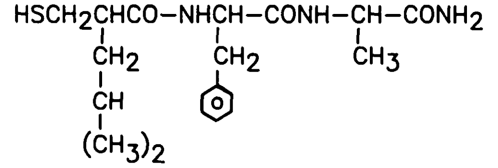
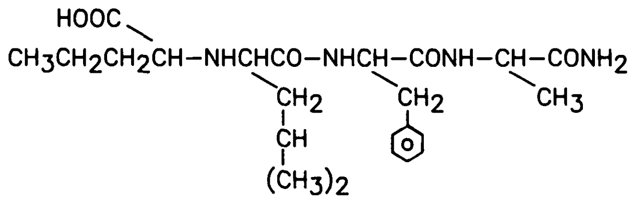


HSCH2–Leu–Phe–Ala–NH2 (Thiol Peptide)



2–KPA–NH–Leu–Phe–Ala–NH2 (Carboxyl Peptide)

Fig. 1. Synthetic peptides used in this study. The top structure is the β-mercaptomethyl tripeptide A. The bottom structure is the N-carboxyalkyl tripeptide B, which is the carboxyl analogue of the thiol peptide. Both structures have been previously demonstrated to be potent inhibitors of nonocular sources of collagenase.

type I collagen as substrate. For quantitative evaluation of inhibitory potency, a newly developed synthetic peptide substrate was used.[21] Additionally, we examined the potential inhibitory properties of sodium citrate and sodium ascorbate since these compounds have been shown to be effective in the management of the alkali-burned eye.

Materials and Methods

The methods of this study were in accord with the ARVO Resolution on the Use of Animals in Research. Intact corneas were excised from albino rabbits approximately 3 weeks after one eye had been burned with 1N NaOH for 60 sec.[3] Corneas were minced and cultured at 37°C in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) that contained 25 mM Hepes, 4.5 g/l glucose, 3.7 g/l $NaHCO_3$, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 ng/ml amphotericin B and 10 ng/ml phorbol 12-myristate 13-acetate.[22] Conditioned medium was removed at intervals of 2–3 days for up to 2 weeks and stored at −20°C. The pooled medium was treated with ammonium sulfate and the fraction that precipitated between 20 and 60% of saturation was dialyzed against 50 mM Hepes, 10 mM $CaCl_2$, 0.05% Brij 35, 0.02% NaN_3, pH 7.2. The latent collagenase was activated with 4-aminophenylmercuric acetate,[23] followed by exhaustive dialysis against the above Hepes buffer. Collagenase and gelatinase were separated from other components by affinity chromatography as described by Stack and Gray.[21] Collagenase was then separated from gelatinase by chromatography on DEAE-Sephacel. The purified collagenase catalyzed formation of the characteristic TC^A and TC^B fragments[24] from type I collagen and was inhibited by EDTA and o-phenanthrolene[25] as expected for a Ca^{2+}-dependent zinc metalloproteinase.

Peptide Inhibitors

The two peptide inhibitors shown in Figure 1 were synthesized and characterized as described previously.[19,26] In the case of the thiol tripeptide A, the diastereomers were resolved by C_{18}-reversed phase HPLC and the more slowly eluting isomer was used. It was dissolved in either dimethyl sulfoxide or 95% ethanol containing 1 mM acetic acid immediately before use and the thiol titer was determined using the Ellman procedure.[27] The N-carboxyalkyl tripeptide B was obtained as a mixture of approximately equal amounts of each diastereomer. The trifluoroacetate salt was dissolved in water and titrated to pH 7 with NaOH.

Other Reagents

Acid-soluble calf skin collagen, N-acetyl-L-cysteine, tetracycline, minocycline, doxycycline, phorbol 12-myristate 13-acetate and L-cysteine were from Sigma Chemical Co. L-Ascorbic acid was from Matheson, Coleman and Bell (Norwood, OH). Citric acid was from Fisher (Fair Lawn, NJ). Stock solutions of nonpeptide inhibitors were freshly prepared in water and brought to pH 7 if necessary with NaOH before use.

Assays

Collagenase activity was determined using type I collagen (0.4 mg/ml) in 0.05 M Tris-HCl, 0.2 M NaCl, 10 mM $CaCl_2$, 0.25 M glucose (to prevent fibril formation), pH 7.7.[28] Reactions were initiated by adding enzyme and were incubated for 3 hr at 30°C in the presence or absence of the test compound. Where indicated, excess $CaCl_2$ was added. Reactions were quenched by placing on ice and then adding 1 volume of sample dilution buffer[29] followed by placing in a boiling water bath for at least 5 min. Collagen degradation products were resolved from undegraded collagen by sodium dodecylsulfate-polyacrylamide gel electrophoresis[29] (SDS-PAGE) followed by staining with Coomassie Blue R250.[19] The SDS-PAGE assay was used to evaluate qualitatively the inhibitory capacity of the compounds tested. An estimate of inhibitory potency was then noted by visualizing the concentration range which produced 50% inhibition of collagen degradation. These estimated ranges were then used to predict the inhibitor concentrations required for the quantitative assay described below.

Table 1. Inhibitor potencies by SDS-PAGE

Inhibitor	*Estimated IC_{50} range (mM)*
I Thiol synthetic peptide	0.001-0.003
II Carboxyl synthetic peptide	0.1-0.3
III Sodium citrate*	10-30
IV Cysteine	3-10
V Acetylcysteine	10-30
VI Tetracycline	1-2
VII Doxycycline	0.2-1
VIII Sodium ascorbate	No inhibition

* Reversed by addition of excess Ca^{2+}.

Quantitative determination of collagenase activity was carried out with a fluorogenic peptide substrate, Dnp-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2, which collagenase cleaves to produce Dnp-Pro-Leu-Gly and Leu-Trp-Ala-D-Arg-NH_2.[21] The rate of production of the tetrapeptide was monitored with an Aminco-Bowman spectrofluorometer (excitation wavelength of 280 nm and emission wavelength of 346 nm). Compounds that absorbed light in the exciting or emission regions of the spectrum, such as the tetracyclines, interfered with the fluorometric assay through the inner filter effect. This problem was obviated by conducting assays of the tetracyclines by HPLC separation of products.[30] Reaction mixtures for both the fluorometric and HPLC assays contained 0.05 M Tris-HCl, 0.2 M NaCl, 10 mM $CaCl_2$ and DNP-substrate 20 μM, pH 7.7. Inhibitors were added to the assay mixtures at varying concentrations. The incubation conditions differed between the two assays; the fluorometric reaction mixtures were incubated at 37°C for 2 to 3 min whereas the HPLC reaction mixtures were incubated at 37°C for 3 hr. This was necessary because the HPLC assay was not as sensitive in detecting the substrate cleavage products as the fluorometric assay. IC_{50} values were interpolated from plots of $\log[(A_0/A_i)-1]$ vs. log[Inhibitor], where A_0 is the activity observed in the absence of inhibitor and A_i is the activity observed in the presence of inhibitor at concentration i.[31]

Results

Except for ascorbic acid, all of the compounds tested inhibited rabbit corneal collagenase to some degree. By far the most potent inhibitors were found to be the synthetic peptides. These peptides demonstrated significantly lower IC_{50} values in comparison to the other compounds tested. It was also shown that sodium citrate inhibited corneal collagenase and that this inhibition was reversed by the addition of excess calcium.

Tables 1, 2 and 3 summarize the IC_{50} values for each compound tested. Table 1 represents IC_{50} value ranges estimated from SDS-PAGE assays. The ranges of IC_{50} values were obtained by visualizing the inhibitor concentration range that produced 50% inhibition of collagen degradation. Table 2 shows results obtained by using the synthetic substrate with the fluorometric assay and Table 3 shows results determined by using the synthetic substrate with the HPLC assay.

Table 2. Inhibitor potencies by fluorometric assay

Inhibitor	*IC_{50}*
I Thiol synthetic peptide	11 nM
II Carboxyl synthetic peptide	25 μM
III Sodium citrate	45 mM

Synthetic Peptides

The effect of the synthetic peptides upon collagenase was initially evaluated by SDS-PAGE. This gave an estimation of their inhibitory effect upon the corneal collagenase. The IC_{50} estimates obtained indicated that the carboxyl peptide had an estimated IC_{50} range of 0.1 to 0.3 mM and the thiol peptide had an estimated IC_{50} range of 0.001 to 0.003 mM.

IC_{50} values, determined by using the synthetic substrate, are shown in Figures 2, 3 and 4 and Tables 2 and 3. Figures 2 and 3 show the results of the HPLC assay demonstrating the potency of the thiol and carboxyl peptides in comparison with the other compounds. The IC_{50} of the thiol peptide by this assay was 100 nM and the IC_{50} of the carboxyl peptide was 22 μM. Figure 4 shows the results of the fluorometric assay comparing the thiol and carboxyl peptides. These data indicate that the carboxyl peptide had an IC_{50} of 25 μm and the thiol peptide had an IC_{50} of 11 nM.

Tetracycline Compounds

The three tetracycline compounds were evaluated by both SDS-PAGE and HPLC assays. Table 1 shows the IC_{50} ranges for doxycycline and tetracycline. The IC_{50} for doxycycline was 0.2 to 1.0 mM and the IC_{50} for tetracycline was 1.0 to 2.0 mM. When evaluating

Table 3. Inhibitor potencies by HPLC assay

Inhibitor	*IC_{50}*
I Thiol synthetic peptide	100 nM
II Carboxyl synthetic peptide	22 μM
III Doxycycline	15 μM
IV Minocycline	190 μM
V Tetracycline	350 μM
VI Cysteine	370 μM
VII Acetylcysteine	2.7 mM
VIII Ascorbic acid	No inhibition

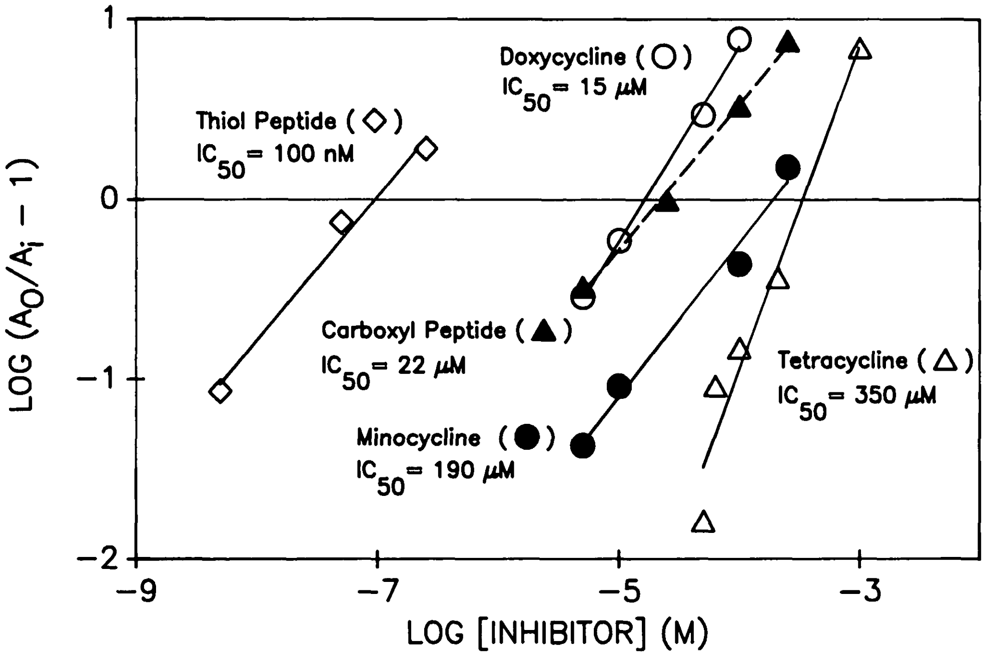


Fig. 2. Effect of the thiol and carboxyl synthetic peptides and the tetracycline compounds upon the activity of rabbit corneal collagenase. This plot demonstrates the significantly higher potency of the thiol peptide (◇) in comparison to three tetracycline compounds: Tetracycline (△), minocycline (●) and doxycycline (O). The carboxyl peptide (▲) was shown to have a potency similar to doxycycline. These compounds were evaluated with the synthetic substrate by HPLC (see text). IC_{50} values were interpolated from plots of log $[(A_0/A_i)-1]$ vs. log [Inhibitor], where A_0 is the activity observed in the absence of inhibitor and A_i is the activity observed in the presence of inhibitor at concentration i; lines were drawn by linear regression analysis. Note that doxycycline is the most potent collagenase inhibitor of the tetracycline compounds tested. Also, note that the thiol peptide is 150 times more potent than doxycycline by this assay.

these same compounds with the synthetic substrate assay, the IC_{50} values shown in Table 3 were obtained; these values were derived from the graph in Figure 2 as described in the legend of Figure 2. Doxycycline was shown to be the most potent inhibitor of the tetracycline compounds with an IC_{50} of 15 μm compared to minocycline (190 μM) and tetracycline (350 μm).

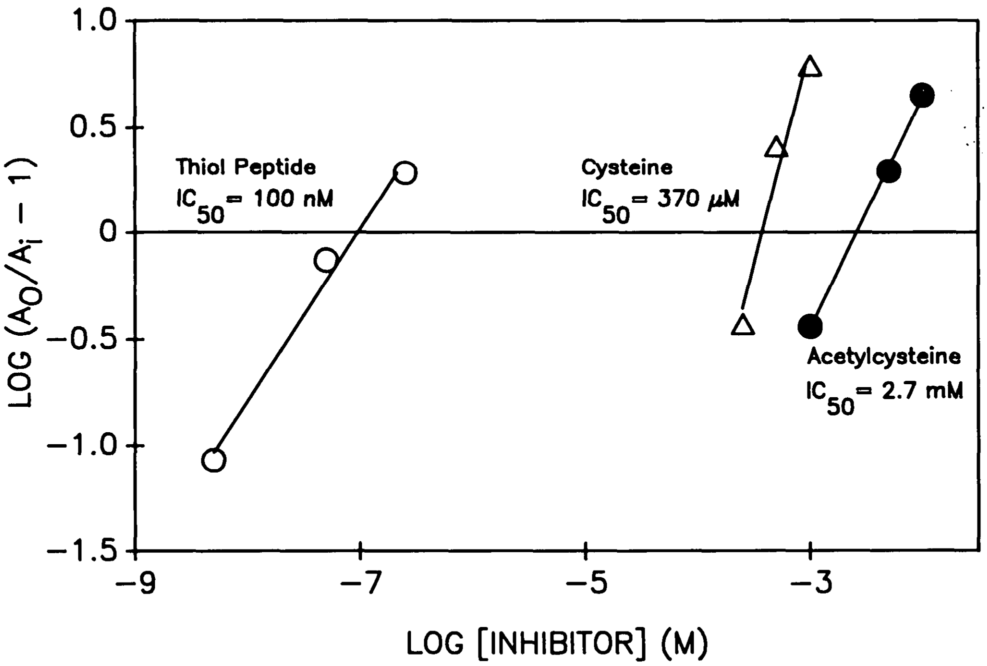


Fig. 3. Effect of thiol compounds upon the activity of rabbit corneal collagenase. This plot demonstrates that the thiol synthetic peptide (O) is much more potent in inhibiting corneal collagenase than two other thiol-containing collagenase inhibitors, cysteine (△) and acetylcysteine (●). These compounds were evaluated with the fluorogenic substrate by HPLC (see text). IC_{50} values were interpolated as described in the legend of Figure 2. Lines were drawn by linear regression analysis. Note that cysteine is approximately 7 times more potent than acetylcysteine. Also note that the thiol synthetic peptide is over 3000 times more potent that cysteine and is 2.7×10^4 times more potent than acetylcysteine by this method.

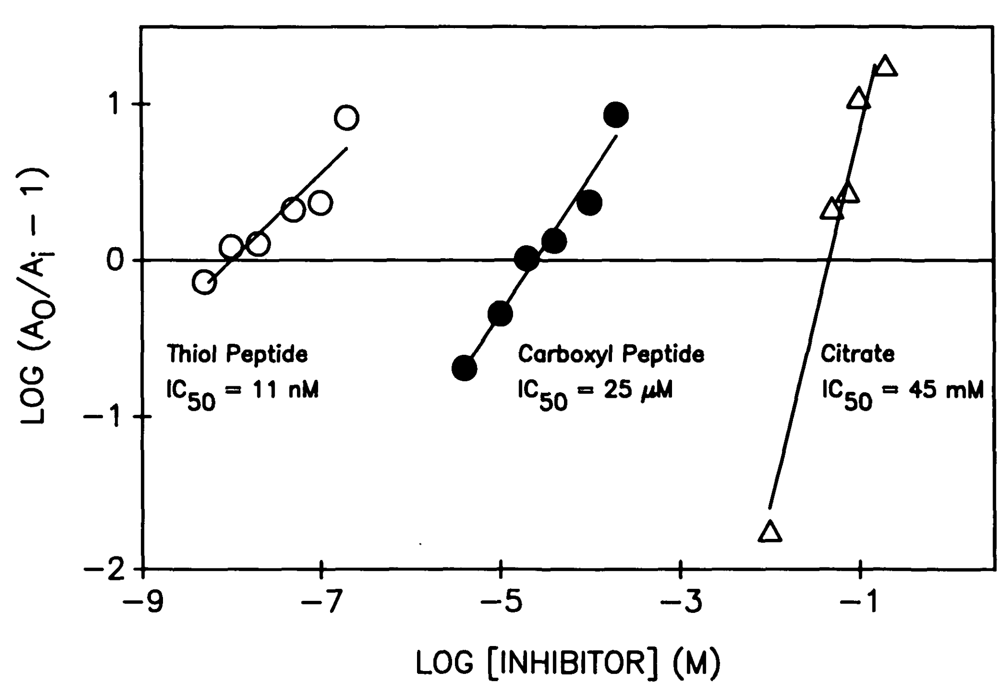


Fig. 4. Effect of the synthetic peptides and sodium citrate upon the activity of rabbit corneal collagenase. This plot demonstrates that the thiol synthetic peptide (○) is much more potent than its carboxyl analogue (●) and sodium citrate (△). These compounds were evaluated with the fluorogenic substrate by spectrofluorometric analysis (see text). IC_{50} values were interpolated as described in the legend of Figure 2. Lines were drawn by linear regression analysis. Note that the carboxyl peptide is nearly 2000 times more potent than sodium citrate. Also note that the thiol peptide is over 2000 times more potent than the carboxyl peptide and more than 4×10^6 times more potent than sodium citrate by this method.

Sodium Citrate and Sodium Ascorbate

Sodium citrate was found to inhibit the collagenase with an estimated IC_{50} range between 10 and 30 mM by SDS-PAGE, as shown in Table 1. Figure 4 shows results obtained by using the fluorometric assay; citrate was found to have an IC_{50} of 45 mM, as shown in Table 2, in comparison to the values for the synthetic peptides. The inhibition of collagenase by citrate was reversed by the addition of excess calcium (25 mM) in the SDS-PAGE assay.

Sodium ascorbate showed no inhibition of the rabbit corneal collagenase either by using collagen as the substrate or the synthetic substrate.

Acetylcysteine and Cysteine

Acetylcysteine was shown to inhibit the corneal collagenase with an estimated IC_{50} range of 10 to 30 mM by SDS-PAGE, as shown in Table 1. Cysteine was found to have an estimated IC_{50} range of 3 to 10 mM by SDS-PAGE, as shown in Table 1. Figure 3 and Table 3 demonstrate results obtained by testing cysteine and acetylcysteine with the synthetic substrate via the HPLC assay.

Discussion

Alkali burns to the eye can cause devastating and permanent ocular damage, frequently causing reduction in visual acuity or loss of the eye. These injuries can result in prolonged or total disability and can create complex management problems for the clinician.[1] Many treatment approaches have been used in treating alkali injuries and these have been reviewed thoroughly.[1,2] The pivotal involvement of collagenase to the development of corneal ulcers has been well acknowledged.[6-8] Therefore, an obvious target of therapy has been to inhibit the release or action of collagenase. The efficacy of currently available inhibitors of collagenase in the treatment of alkali burns is open to question.[1] Results of the current study clearly indicate that a new group of synthetic peptides have a significant impact upon corneal collagenase in vitro.

We have recently developed a number of synthetic collagenase inhibitors.[18-20] The mechanism by which they function is presumably by binding to the active site of the enzyme and coordinating with Zn^{2+} at that site.[32] It has been shown that these compounds are effective in inhibiting nonocular sources of collagenase (ie, pig synovial collagenase and rabbit V-2 tumor collagenase).[19,20] Previous results have indicated that thiol-containing inhibitors, developed as analogues of the carboxyl side of the collagenase-sensitive bond of collagen, were far more potent than their N-carboxyalkyl counterparts.[19,20]

The findings of the current study have shown that both the carboxyl and thiol peptides tested were potent inhibitors of collagenase derived from the cornea. It was also demonstrated that the thiol peptide was far more potent than any other compound tested including the carboxyl peptide. This finding held true with all three assays used during the study. Although

IC_{50} values differed for the compounds when comparing between the various assays, the order of inhibitor potency was always the same. The variations in IC_{50} values were probably due to the longer incubation times for the SDS-PAGE and HPLC assays, thus allowing autooxidation of the essential −SH moiety of the thiol peptide to occur. The presence of 0.25 M glucose in the SDS-PAGE assay and the different incubation temperatures (30° vs. 37°C) may have also affected the IC_{50} values obtained.

Three different tetracycline compounds were tested with the corneal collagenase. The most potent inhibitor of this group was found to be doxycycline. It has been suggested that the mechanism by which the tetracyclines inhibit collagenase is by the chelation of calcium.[13–17] Under most conditions, inhibition occurred when the antibiotic concentration was much lower than the Ca^{2+} concentration. Thus, it is very unlikely that the antibiotic would bind enough calcium to cause collagenase inhibition. Therefore, there is some question as to whether this is the mechanism by which these antibiotics inhibit collagenase. It is more likely that tetracyclines bind essential Zn^{2+} in collagenase and, thus, inhibit by this mechanism. Previous studies have shown that the tetracyclines bind Zn^{2+} and that doxycycline binds Zn^{2+} more tightly than the other tetracyclines.[33] This may explain why doxycycline is a more potent inhibitor of collagenase than minocycline or tetracycline.

Large numbers of polymorphonuclear leukocytes (PMNs) which contain numerous degradative enzymes are found in the cornea following an alkali burn. This has lead to the implication that PMNs play a very prominent role in corneal ulcer formation.[34] Pfister et al have shown that citrate has enormous inhibitory effects upon PMNs.[35] Our findings indicate that in addition to citrate's effect on PMNs, it also inhibits the action of collagenase. Although it is quite clear that citrate has a significant impact upon PMN activation and locomotion, it should not be overlooked that citrate also has the potential to inhibit collagenase in the alkali-burned cornea. Ascorbate demonstrated no inhibitory effect upon the corneal collagenase. This finding suggests that the beneficial action of ascorbate in treating alkali burns is not due to inhibition of collagenase.

Acetylcysteine and cysteine have also been shown to be effective in inhibiting the incidence of corneal ulceration in experimental alkali burns.[9,10] Both compounds presumably bind to active-site Zn^{2+} as their mechanism of inhibiting collagenase.[36,37] Since their potency is rather weak, relatively high concentrations must be used to be effective in vivo.[9,10] Our studies have shown that the two compounds share similar potencies but that they both are far weaker inhibitors than the synthetic peptides which were tested.

In considering the biochemical and physiologic events that occur following a corneal alkali injury, the need for a combined approach to treatment becomes quite obvious. First, by considering that severely reduced levels of ascorbic acid are found in the aqueous humor following an alkali burn, which causes a local scorbutus and a reduction in the local production of collagen, a means of increasing the ascorbate level is necessary. This could be accomplished by systemic and topical ascorbate treatment. Second, because of the large influx of inflammatory cells, mostly PMNs, an effective inhibitor of PMN migration and activation is needed; citrate has been shown to accomplish this.[35] Finally, an inhibitor of collagenase to inhibit enzyme which is released from non-PMN sources, such as fibroblasts, and also from PMNs which may evade citrate, is required. The results of this study suggest that the thiol peptide might be a good candidate for this aspect of treatment. By providing an effective multifaceted approach to the treatment of the alkali-burned eye, it is felt that the integrity of the eye will be more readily preserved and the eventual clinical outcome will be significantly enhanced.

Key words: corneal ulceration, collagenase inhibitors, alkali burns, collagen, thiol peptides

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References

1. McCulley JP: Chemical injuries. *In* The Cornea: Scientific Foundations and Clinical Practice, Smolin G and Thoft RA, editors. Boston, Little, Brown and Co., 1983, pp. 422–435.
2. Pfister RR: Chemical corneal burns. Int Ophthalmol Clin 24:157, 1984.
3. Levinson R, Paterson CA, and Pfister RR: Ascorbic acid prevents corneal ulceration and perforation following experimental alkali burns. Invest Ophthalmol 15:986, 1976.
4. Pfister RR, Paterson CA, and Hayes SA: Topical ascorbate decreases the incidence of corneal ulceration after experimental alkali burns. Invest Ophthalmol Vis Sci 17:1019, 1978.
5. Pfister RR, Nicolaro ML, and Paterson CA: Sodium citrate reduces the incidence of corneal ulcerations and perforations in extreme alkali-burned eyes—acetylcysteine and ascorbate have no favorable effect. Invest Ophthalmol Vis Sci 21:486, 1981.
6. Brown SI, Weller CA, and Wasserman HE: Collagenolytic activity of alkali-burned corneas. Arch Ophthalmol 81:370, 1969.
7. Berman MB, Dohlman CH, Gnadinger M, and Davison P: Characterization of collagenolytic activity in the ulcerating cornea. Exp Eye Res 11:255, 1971.

8. Gordon JM, Bauer EA, and Eisen AZ: Collagenase in human cornea: Immunologic localization. Arch Ophthalmol 98:341, 1980.
9. Slansky HH, Berman MB, Dohlman CH, and Rose J: Cysteine and acetylcysteine in the prevention of corneal ulcerations. Ann Ophthalmol 2:488, 1970.
10. Brown SI, Akiya S, and Weller CA: Prevention of the ulcers of the alkali-burned cornea: Preliminary studies with collagenase inhibitors. Arch Ophthalmol 82:95, 1969.
11. Brown SI and Weller CA: The pathogenesis and treatment of collagen-induced diseases of the cornea. Trans Am Acad Ophthalmol Otolaryngol 74:375, 1970.
12. Francois J, Cambie E, Feher J, and Van Den Eeckhout E: Collagenase inhibitors (penicillamine). Ann Ophthalmol 5:391, 1973.
13. Golub LM, Lee HM, Lehrer G, Nemiroff A, McNamara TF, Kaplan R, and Ramamurthy NS: Minocycline reduces gingival collagenolytic activity during diabetes: Preliminary observations and a proposed new mechanism of action. J Periodont Res 18:516, 1983.
14. Golub LM, Ramamurthy N, McNamara TF, Gomes B, Wolff M, Casino A, Kapoor A, Zambon J, Ciancio S, Schnier M, and Perry H: Tetracyclines inhibit tissue collagenase activity: A new mechanism in the treatment of periodontal disease. J Periodont Res 19:651, 1984.
15. Golub LM, Wolff M, Lee HM, McNamara TF, Ramamurthy NS, Zambon J, and Ciancio S: Further evidence that tetracyclines inhibit collagenase activity in human crevicular fluid and other mammalian sources. J Periodont Res 20:12, 1985.
16. Lee HM, Golub LM, Gwinnett AJ, and Ramamurthy NS: Minocycline inhibits collagenase and elastase produced by rat macrophages in cell culture. J Dent Res (Special Issue) 64:305, 1985.
17. Seedor JA, Perry HD, McNamara TF, Golub LM, Buxton DF, and Guthrie DS: Systemic tetracycline treatment of alkali-induced corneal ulceration in rabbits. Arch Ophthalmol 105:268, 1987.
18. Gray RD, Saneii HH, and Spatola AF: Metal binding peptide inhibitors of vertebrate collagenase. Biochem Biophys Res Commun 101:1251, 1981.
19. Gray RD, Miller RB, and Spatola AF: Inhibition of mammalian collagenase by thiol-containing peptides. J Cell Biochem 32:71, 1986.
20. Gray RD, Darlak K, Miller RB, Stack MS, and Spatola AF: Inhibition of mammalian collagenase by thiol and N-carboxyalkyl peptide derivatives. FASEB J 2:A345 (Abstract), 1988.
21. Stack MS and Gray RD: Comparison of vertebrate collagenase and gelatinase using a new fluorogenic substrate peptide. J Biol Chem 264:4277, 1989.
22. Brinckerhoff CE, McMillan RM, Faley JV, and Harris ED Jr: Collagenase production by synovial fibroblasts treated with phorbol myristate acetate. Arthritis Rheum 22:1109, 1979.
23. Cawston TE and Tyler JA: Purification of pig synovial collagenase to high specific activity. Biochem J 183:647, 1979.
24. Gross J and Lapiere CM: Collagenolytic activity in amphibian tissues: A tissue culture assay. Proc Natl Acad Sci USA 48:1014, 1962.
25. Harper E and Seifter S: Studies on the mechanisms of action of collagenase: Inhibition by cysteine and other chelating agents. Israel J Chem 12:515, 1974.
26. Gray RD, Pierce WM Jr, Harrod JS Jr, and Rademacher JM: Inhibition of thermolysin by bifunctional N-carboxyalkyl dipeptides. Arch Biochem Biophys 256:692, 1987.
27. Ellman GL: Tissue sulfhydryl groups. Arch Biochem Biophys 82:70, 1959.
28. Terato K, Nagai Y, Kawahishi K, and Yamamoto S: A rapid assay method of collagenase activity using ^{14}C-labelled soluble collagen as substrate. Biochem Biophys Acta 445:753, 1976.
29. Laemmli UK: Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227:680, 1970.
30. Gray RD and Saneii HH: Characterization of vertebrate collagenase activity by high performance liquid chromatography using a synthetic substrate. Ann Biochem 120:339, 1982.
31. Ambrose JF, Kistiakowski GB, and Kridl AG: Inhibition of urease by S compounds. J Am Chem Soc 72:317, 1950.
32. Cushman DW, Cheung HS, Sabo EF, and Ondetti MA: Design of potent competitive inhibitors of angiotensin-converting enzyme: Carboxyalkanoyl and mercaptoalkanoyl amino acids. Biochemistry 16:5484, 1977.
33. Brion M, Lambs L, and Berthon G: Metal ion-tetracycline interactions in biological fluids: Part 5. Formation of zinc complexes with tetracycline and some of its derivatives and assessment of their biological significance. Agents Actions 17:229, 1985.
34. Kenyon K, Berman M, Rose J, and Gage J: Prevention of stromal ulceration in the alkali-burned rabbit cornea by glued-on contact lens: Evidence for the role of polymorphonuclear leukocytes in collagen degradation. Invest Ophthalmol Vis Sci 18:570, 1979.
35. Pfister RR, Haddox JL, Dodson RW, and Deshazo WF: Polymorphonuclear leukocytic inhibition by citrate, other metal chelators and trifluoperazine: Evidence to support calcium binding protein involvement. Invest Ophthalmol Vis Sci 25:955, 1984.
36. Berman M and Dohlman C: Collagenase inhibitors. Arch Ophthalmol 35:95, 1975.
37. Berman M and Manabe R: Corneal collagenases: Evidence for zinc metalloenzymes. Ann Ophthalmol 5:1193, 1973.