*Free Rad. Res.*, Vol. 28, pp. 115–123
Reprints available directly from the publisher
Photocopying permitted by license only





# The Oxidative Inactivation of Tissue Inhibitor of Metalloproteinase-1 (TIMP-1) by Hypochlorous Acid (HOCl) is Suppressed by Anti-Rheumatic Drugs

FARIBA SHABANI*, JULIAN MCNEIL and LINDA TIPPETT

*Department of Medicine Royal Adelaide Hospital and Modbury Public Hospital, and Department of Chemistry, University of Adelaide, Adelaide, South Australia, 5000*

Accepted by Prof. B. Halliwell

*(Received 16 July 1997; In revised form 5 September 1997)*

Tissue inhibitors of metalloproteinases (TIMPs) prevent uncontrolled connective tissue destruction by limiting the activity of matrix metalloproteinases (MMPs). That TIMPs should be susceptible to oxidative inactivation is suggested by their complex tertiary structure which is dependent upon 6 disulphide bonds. We examined the oxidative inactivation of human recombinant TIMP-1 (hr TIMP-1) by HOCl and the inhibition of this process by anti-rheumatic agents.

TIMP-1 was exposed to HOCl in the presence of a variety of disease modifying anti-rheumatic drugs. TIMP-1 activity was measured by its ability to inhibit BC1 collagenase activity as measured by a fluorimetric assay using the synthetic peptide substrate (DNP-Pro-Leu-Ala-Leu-Trp-Ala-Arg), best cleaved by MMP-1.

The neutrophil derived oxidant HOCl, but not the derived oxidant N-chlorotaurine, can inactivate TIMP-1 at concentrations achieved at sites of inflammation. Anti-rheumatic drugs have the ability to protect hrTIMP-1 from inactivation by HOCl. For D-penicillamine, this effect occurs at plasma levels achieved with patients taking the drug but for other anti-rheumatic drugs tested this occurs at relatively high concentrations that are unlikely to be achieved in vivo, except possibly in a microenvironment. These results are in keeping with the concept that biologically derived oxidants can potentiate tissue damage by inactivating key but susceptible protein inhibitors such as TIMP-1 which form the major local defence against MMP induced tissue breakdown.

*Keywords:* Tissue inhibitor of metalloproteinases (TIMP), oxidants, anti-rheumatic drugs and hypochlorous acid

## INTRODUCTION

The matrix metalloproteinases (MMPs) are a family of proteolytic enzymes that are capable of degrading the macromolecules of the extracellular matrix. They represent an important mechanism by which the turnover of structural proteins such as collagen is regulated.[1] MMP-1 can cleave native collagen molecules and allow the resultant denatured collagen to be digested by a range of other enzymes including other MMPs. In this way MMP-1 regulates fibrillar collagen turnover.[2,3]

* Corresponding author. Tel.: (618)82224320. Fax: (618)82233870.

Tissue inhibitors of metalloproteinases (TIMPs) form a family of proteins and are the major local inhibitors of MMPs with activity against all known MMPs.[4] TIMPs regulate the activity of MMPs by tightly binding to the active site and forming inactive complexes with a 1 : 1 stoichiometry.[5] The balance between the activities of MMPs and TIMPs is a crucial factor in regulating extracellular matrix breakdown in vivo.[6] Thus far, four members of the TIMP family have been identified, namly TIMP-1, TIMP-2, TIMP-3 & TIMP-4. TIMP-1 is a 30 KDa glycoprotein found in most body fluids. TIMP-2 is an unglycosylated 21 KDa protein. These two proteins have 40% amino acid sequence homology. Average concentrations of MMPs and TIMP fragments are significantly elevated in the joint fluid of patients with osteoarthritis (OA) as compared with volunteers with healthy knees.[7] Structural studies of TIMP-1 suggest a highly conserved secondary structure. There are 12 cysteine residues which form six conserved disulfide bonds, giving a protein structure of six loops and two domains. TIMP activity is dependent upon this elaborate tertiary structure.[8] The high density of disulfide bonds and relatively complicated tertiary structure, which is a functional requirement, would suggest that this proteinase inhibitor is susceptible to oxidants.[6]

HOCl is a major product of the oxidative burst of neutrophils.[9] There is accumulation of polymorphonuclear leukocytes (PMN) in the synovial fluid of patients with rheumatoid arthritis (RA) which, after stimulation, may release inflammatory mediators.[10] The oxidative burst of neutrophils generates superoxide anion ($O_2^{\bullet -}$), which is rapidly dismutated to form hydrogen peroxide ($H_2O_2$). The neutrophil enzyme, myeloperoxidase, catalyzes the reaction of $H_2O_2$ with chloride ions to form the highly reactive and cytotoxic agent hypochlorous acid (HOCl).[11] In aqueous solution HOCl exists in essentially equal concentration as the conjugate base hypochlorite ($OCl^-$) and together form a powerful biologically relevant oxidizing agent capable of causing damage to proteins.[12]

HOCl will react with amino acids such as taurine to generate a derivative group of oxidants known as the chloramines.[13] Generally, chloramines are less powerful oxidants than HOCl itself.[9] Taurine, whose biological role remains unclear, is found at concentrations approaching 20 mM in neutrophils and macrophages.[14] N-Chlorotaurine has been reported to be essentially unreactive with major cellular components. It has been suggested that taurine may act as a "sink" for the oxidizing potential of HOCl/$OCl^-$, generating a chlorinated species of lower reactivity and thereby minimizing indiscriminate damage.[14] Since taurine is the most abundant endogenous amine in the pericellular environment of the neutrophil,[14] we have used N-chlorotaurine as an example of chloramines in our model. In many cases oxidation appears to result in a partial unfolding or rearrangement of target proteins.[15,16]

Previous reports have shown that latent neutrophil collagenase (MMP-8) can be activated by neutrophil derived oxidants, principally HOCl.[9,11,12] This has highlighted the way that oxidant generation and enzymatic mechanisms can interact to lead to tissue breakdown. Direct oxidative inactivation of enzyme inhibitors is a more direct mechanism of interaction between oxidant generation and the equilibrium between proteases and their inhibitors. The most well known instance, where this occurs, is the oxidative inactivation of plasma proteinase inhibitors, such as $\alpha_1$-proteinase inhibitor ($\alpha_1$-PI), where oxidation of the methionine at the active site of $\alpha_1$-PI results in loss of affinity of this inhibitor for its target enzyme.[17] In another study by Frears *et al.* the inactivation of TIMP-1 by peroxynitrite ($ONOO^-$) has been demonstrated.[18] We have reasoned that since hrTIMP-1 has an elaborate 6 loop structure, each loop maintained by interchain disulfide bonds, that oxidative cleavage of these disulfide bonds would result in major structural disintegration and consequent loss of affinity for matrix metalloproteinases.

Thiol-containing antirheumatic drugs, such as D-penicillamine, sodium aurothiomalate, and aurothioglucose, are widely used in treatment of RA.[19] Some studies have investigated the effects of antirheumatic drugs, including thiol-containing drugs, on the oxidative activity of myeloperoxidase.[20,21] It has been shown that D-penicillamine effectively scavenges HOCl formed by myeloperoxidase and inhibits the enzyme itself.[17]

In this study, we have examined the possibility that neutrophil derived oxidants may inactivate hrTIMP-1 and thus allow MMPs to be unfettered in areas of HOCl generation, as for example the pericellular environment of the activated neutrophil. We have also examined the hypothesis that anti-rheumatic drugs, particularly those shown to act as anti-oxidants, may protect hrTIMP-1 from oxidative inactivation.[20]

## MATERIALS AND METHODS

### Materials

D-Penicillamine, gold sodium thioglucose (aurothioglucose), chloroquine, taurine and Tris (hydroxymethyl aminomethane) [Tris], were purchased from the Sigma Chemical Company, St. Louis, MO., USA. Gold sodium thiomalate (aurothiomalate) was from Aldrich, U.K. Reagent grade chemicals were purchased from BDH Chemicals Australia Pty. Ltd., Kilsyth, Victoria, Australia. Media were from Cytosystems Pty Ltd, Castle Hill, NSW, Australia. Auranofin was from Smith, Klein and French (Pty Ltd). Methotrexate was from David Bull Laboratories, Melbourne, Australia. Aurothiomalate and aurothioglucose were stored desiccated as powder at −20°C. D-penicillamine, methotrexate, chloroquine and auranofin were stored desiccated at 4°C.

Human recombinant TIMP-1 (hrTIMP-1) was a gift from Dr. A. Docherty (Celltech Research Slough, Berkshire, U.K). The rat mammary carcinoma cell line BC-1 was a gift from Dr. J. O'Grady, University of Technology, Sydney, Australia.[23]

N-chlorotaurine was made by the addition of a solution of NaOCl to taurine buffer (50 mM Taurine, 10 mM $CaCl_2$, 100 mM NaCl, pH 7.5) according to the method of Weiss *et al.*[12]

### BC-1 Collagenase

Serum-free medium (HAMS F-12.10.65g/2L, DMEM 18.42g/2L, $NaHCO_3$ 2g/2L, Pen/Strep 5U/ml, insulin 5μg/ml, transferrin 1mg/ml, L-glutamine 0.292mg/ml, BSA 5mg/ml), was conditioned by BC-1 cells to produce MMP-1 (serum-free-BC-1). These cells have the ability to produce large quantities of matrix metalloproteinases, particularly MMP-1. The enzyme, BC-1 collagenase was purified by passing the conditioned media through Heparin-Sepharose affinity column, and was eluted from the column using an eluting buffer consisting of Tris-Cl 50 mM, NaCl 800 mM, $CaCl_2$ 10 mM, 0.2% $NaN_3$.[23] Column eluates were assayed for protein by using serial concentrations of the bovine serum albumin (BSA) as standard curve and measuring the absorbance at 280 nm.

### Activation of BC-1 Collagenase

Enzyme BC-1 collagenase was incubated at 35°C for 15 mins with trypsin at a final concentration of 2.5 μg/ml in the assay buffer (Tris 50 mM, NaCl 100 mM, $CaCl_2$ 10 mM, $NaN_3$ 0.02%, pH 7.5). The reaction was terminated using 12.5 μg/ml SBTI. A time course for this assay of collagenase activity showed that the maximum activity was achieved between 5–15 mins incubation of the collagenase with trypsin.

### Fluorimetric Assay of Collagenase Activity

This assay was performed according to the method of Netzel-Arnett *et al.*[24] The assay relies on cleavage of the substrate, DNP-Pro-Leu-Ala-Leu-Trp-Ala-Arg at the Ala-Leu bond, thus releasing Trp fluorescence from quenching by the DNP group at the N-terminal region of the

hepta-peptide substrate. The substrate used, is best cleaved by MMP-1. The heptapeptide substrate was synthesized using the solid phase method with Nα-Fmoc-aminoacids. The synthetic peptide was coupled with dinitrofluorobenzene prior to cleavage from the resin. It was purified by HPLC using a Waters C18 Novapak column and subsequently recovered by lyophilization. The hepta-peptide was solubilized in 4% DMSO.

Samples, after dialysis against the appropriate buffer, were activated and then equilibrated at 35°C for 30 seconds in a 5 mm path length quartz cuvette (Starna type 3–5) and the assay started with the addition of the heptapeptide substrate to give a final concentration of 12 μM. The reaction was monitored over time at 35°C in a Perkin-Elmer LS-5 fluorimeter (excitation l = 280 nm, slit width: 10 nm; emission l = 360 nm, slit width: 10 nm). Collagenolytic activity was calculated from the initial linear slope of the curve being expressed as the rate of increase in fluorescence at 360 nm per second.

### Collagenase Inhibition Assay

The inhibitory capacity of hrTIMP-1 was determined using the same spectrophotometric assay as above. In this assay, trypsin activated enzyme was used as the indicator of the hrTIMP-1 inhibitory activity. To establish the dose curve with hrTIMP-1, different concentrations of the hrTIMP-1 between 1 to 50 nM was applied to the active enzyme to make the inactive complex in 30 seconds. Synthetic peptide, 12 μM, was then added to show the % inhibition of the enzyme with the inhibitor. Initially concentrations of HOCl between 0.1 and 200 μM were incubated with hrTIMP-1 at 35°C for 1 hour. A time course for this inhibitory assay showed that maximum inhibition was achieved after 30–60 mins pre-incubation of TIMP-1 with HOCl. Subsequently reactants were added to the cuvette in the following sequence: assay buffer, oxidant-exposed hrTIMP-1, enzyme (BC1 collagenase), substrate (synthetic peptide).

Results were expressed as change in fluorescent intensity per unit time. For experimental samples this was expressed as a percent of control, which was the reaction of activated BC1 collagenase and substrate alone.

### Drugs

For experiments involving anti-rheumatic agents, hrTIMP-1 was pre-treated with a range of anti-rheumatic drugs prior to the addition of HOCl. The sequence of addition was as follows: assay buffer, drug, hrTIMP-1, oxidant, BC1 collagenase, substrate. Fresh solutions of drugs were prepared on a daily basis.

### Statistical Analysis

Each experiment was repeated three times. All results were expressed as the mean ± standard error of the mean (SEM).

## RESULTS

### Inhibition of BC-1 Collagenase by Human Recombinant TIMP-1

BC-1 collagenase was activated by trypsin and the reaction was stopped using SBTI. This activated enzyme was used as the control in the following fluorimetric assays. As shown in Figure 1, hrTIMP-1 was able to inactivate BC-1 collagenase that had been activated by pre-exposure to trypsin. The inhibition was investigated for a maximum hrTIMP-1 concentration of 35 nM, at which the BC1 collagenase activity was reduced to 10% of the control. There was a sigmoidal relationship between the concentration of inhibitor and enzyme activity.

### Oxidative Inactivation of TIMP-1

We examined the loss of activity of hrTIMP-1 to inhibit BC1 collagenase, after pre-exposure to

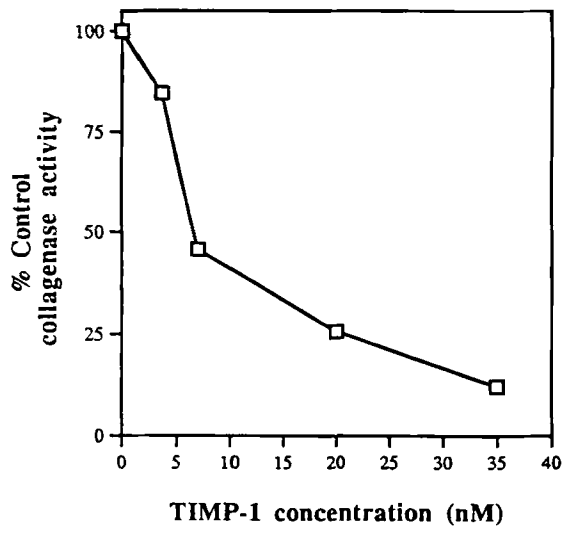


FIGURE 1 Inhibition of trypsin activated BC1 collagenase by TIMP-1. Serial concentrations of TIMP-1 applied to the active collagenase at 37°C. A synthetic peptide was used as the substrate to determine the level of the BC1-collagenase activity. 100% control collagenase activity was the level of BC1-collagenase activity when this enzyme was not treated with any concentration of TIMP-1. 50% inhibition of the BC1-collagenase activity was achieved using 7 nM TIMP-1. Values represent mean ± SEM for three separate experiments expressed as % control collagenase activity. Where SEM is not shown, it is less than or equal to the size of the markers.

HOCl. TIMP-1 was diluted in PBS to give the final concentration of 1.1 μM. Figure 2A shows the inactivation of TIMP-1 after pre-exposure to HOCl between concentrations of 100–200 μM. 100% control TIMP-1 activity is when no HOCl was applied. 50% inactivation was achieved using 175 μM HOCl, while 200 μM HOCl caused complete inactivation of the inhibitor. This represents an oxidant to inhibitor molar ratio of 200:1. In order to show that this was not due to a direct effect of HOCl on BC1 collagenase, HOCl was exposed to BC1 collagenase at the final concentration achieved in this experiment (ie. 200 μM) and no change in collagenase activity was observed (data not shown). When N-chlorotaurine (NCT) was used as an oxidant in concentrations of NCT between (0.1–10 mM) no inactivation of hrTIMP-1 was seen.

A time course of TIMP-1 oxidative inactivation is shown in Figure 2B. TIMP-1 was incubated with HOCl (200 μM) at 37°C for the indicated time points. TIMP-1 oxidative inactivation started after 15 minutes incubation with HOCl reaching to a maximum after 60 minutes.

### Effects of Anti-rheumatic Drugs on BC1 Collagenase Activity

Figure 3 shows the direct effect of several anti-rheumatic agents on BC1 collagenase activity. Auranofin has no inhibitory effect up to a concentration of 500 μM, whereas chloroquine, aurothiomalate and aurothioglucose give moderate inhibition at 500 μM but not 100 μM. Methotrexate and D-penicillamine have significant effects at 100 μM. Subsequent experiments to examine oxidative inactivation of TIMP-1 were therefore designed such that BC1 collagenase used in the collagenase inhibition assay was not exposed to concentrations of each drug greater than 30 μM, where there is no significant effect of the drug on BC1 collagenase activity.

### Effects of Anti-rheumatic Drugs on the Oxidative Inactivation of the TIMP-1

Figure 4 shows the results of experiments where hrTIMP-1 is pre-incubated with a variety of anti-rheumatic drugs prior to HOCl treatment. At 0% TIMP-1 loses its ability to inhibit collagenase when treated with HOCl (200 μM). At 100% TIMP-1 activity, no oxidative inactivation of TIMP-1 is evident as the result of TIMP-1 pre-treatment with some drugs. The first stage of this experiment involves exposure of TIMP-1 to oxidant in the presence of drug to determine whether the drug can protect TIMP-1 from oxidative inactivation. In the second stage of the experiment, TIMP-1, in combination with the drug and HOCl, is exposed to BC1 collagenase to determine TIMP-1 activity. At this stage, because the samples are diluted, BC1 collagenase is not exposured to drug concentrations over 30 μM.

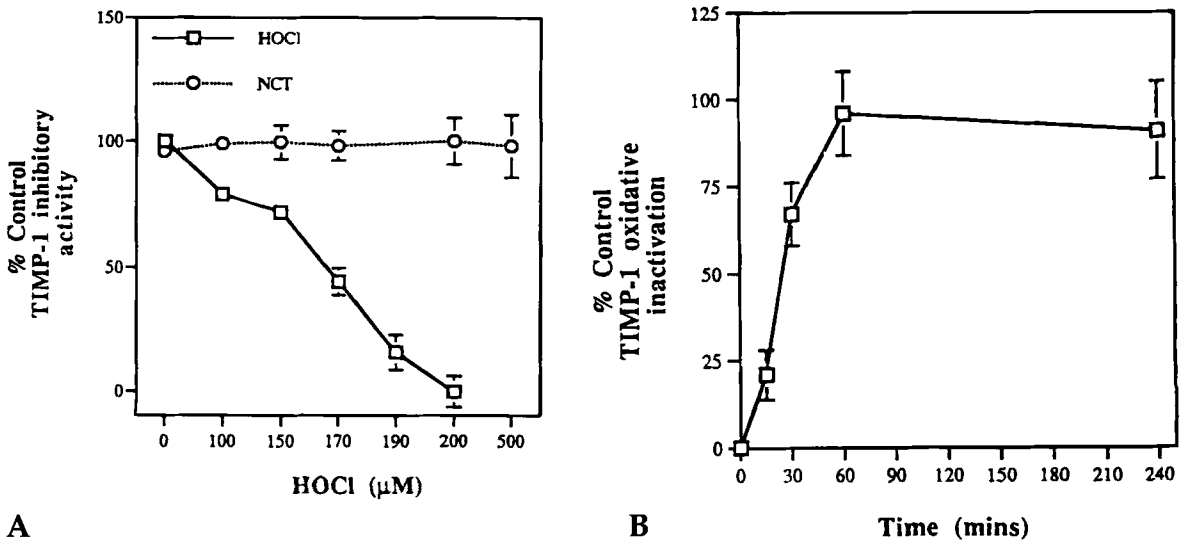


FIGURE 2 Oxidative inactivation of human recombinant TIMP-1 by HOCl. Figure 2A shows, TIMP-1 inactivation with HOCl but not NCT is regulated in a dose dependent manner. TIMP-1 was incubated with increasing concentrations of HOCl (100–200 μM) or NCT (100–500 μM) at 37°C for 1 hour. 100% control TIMP-1 inhibitory activity was the level of TIMP-1 activity when this inhibitor was not incubated with any oxidant. 175 μM HOCl caused 50% inactivation of TIMP-1, whereas no inactivation of TIMP-1 was achieved using NCT up to 500 μM. Using HOCl 200 μM completely abolished the inhibitory activity of TIMP-1. Values represent means ± SEM for three separate experiments expressed as % control TIMP-1 inhibitory activity. TIMP-1 oxidative inactivation was also time dependently regulated. TIMP-1 was incubated with HOCl (200 μM) at 37°C for the indicated time points. According to figure 2B, the oxidative inactivation of TIMP-1 was started after 15 minutes. Maximum activation was acheived after 60 minutes incubation. Values represent means ± SEM for three separate experiments expressed as % control TIMP-1 oxidative inactivation.

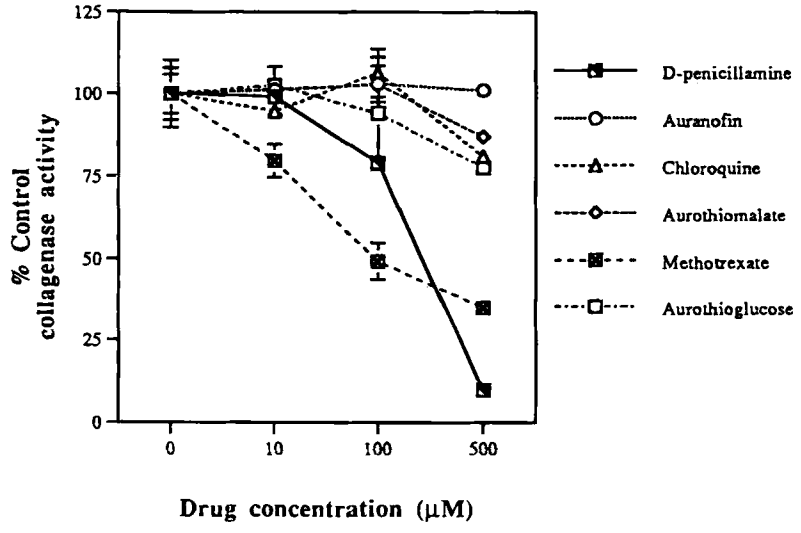


FIGURE 3 Direct effect of several anti-rheumatic agents on BC1-collagenase activity. Trypsin-activated BC1-collagenase was incubated with serial concentrations of different anti-rheumatic drugs including Aurothioglucose (10–500 μM), Aurothiomalate (10–500 μM), Auranofin (10–500 μM), Chloroquine (10–500 μM), D-penicillamine (10–500 μM), and Methotrexate (10–500 μM) for 1 hour at 35°C. 100% control collagenase activity indicates the level of BC1-collagenase activity, when this enzyme was not treated with any drug. Values represent means ± SEM for three separate experiments expressed as % control collagenase activity.

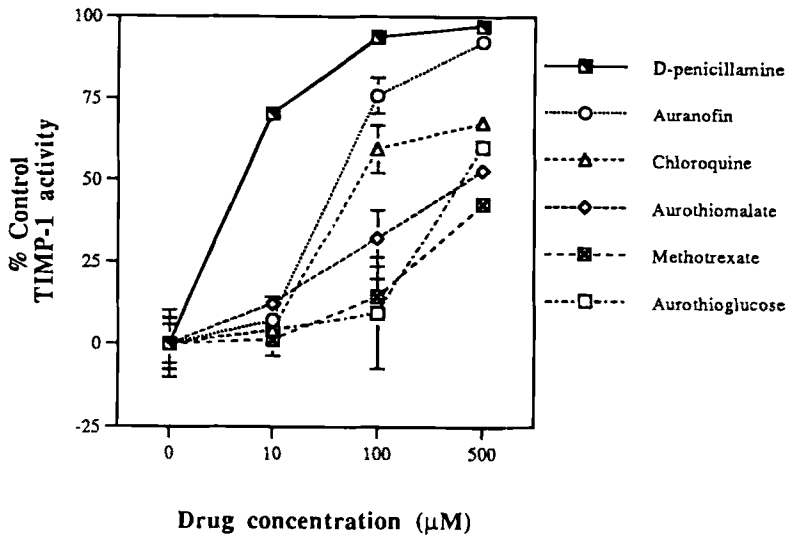


FIGURE 4 Activity of hrTIMP-1 in the prescence of drugs and oxidant. Human recombinant TIMP-1 was pretreated with a variety of anti-rheumatic drugs followed by exposing to HOCl for 1 hour at 35°C. At 0% control TIMP-1 activity, where this inhibitor was not pretreated with drugs, the hrTIMP-1 inhibitory effect against collagenase was completely suppressed by HOCl. Whereas, at 100% control TIMP-1 activity, the oxidative inactivation of hrTIMP-1 was completely abolished by anti-rheumatic drugs. Values represent means ± SEM for three separate experiments expressed as % control TIMP-1 activity.

D-penicillamine shows the greatest ability to protect hrTIMP-1 from oxidative inactivation by HOCl, this effect being evident at a concentration of 10 μM. Of the other drugs tested, auranofin, chloroquine and aurothiomalate had the ability to protect TIMP-1 from oxidative inactivation at a concentration of 100 μM. All drugs were partially effective at the 500 μM concentration, although this is about 20 fold higher than the serum levels acheived during treatment with these agents.

## DISCUSSION

In the present study we have shown that HOCl can directly inactivate TIMP-1 in vitro. TIMP-1 was shown to inhibit the cleavage of a synthetic peptide by an active mammalian MMP(BC1 collagenase). TIMP-1 loses this ability after being pretreated by HOCl. Since HOCl is a product of activated neutrophils, this suggests that neutrophils, which infiltrate inflammatory sites, may contribute to TIMP-1 inactivation. Quantitative analyses have demonstrated that $10^6$ maximally triggered neutrophils produce approximately $2\times10^{-7}$ mol of HOCl during a two-hour incubation.[25] The quantity of oxidants generated by the neutrophil are impressive.

The dependence of the inhibitory activity of TIMP-1 on this 6-loop structure is underlined by its inactivation by reducing and alkylating agents such as dithiothreitol and indoacetamide.[26] Another agent, known to modify the stability of the inhibitor by altering its tertiary structure is guanidine hydrochloride (GdnHCl), resulting in structural disintegration of TIMP-1.[27] The structure of TIMP-1 has also been modified using diethylpyrocarbonate (DEPC), which is a potent inactivator of human TIMP-1. Exposure to DEPC specifically modifies histidine residues, resulting in loss of human TIMP-1 activity.[28]

We demonstrated that pre-exposure of hrTIMP-1 to HOCl resulted in loss of its capacity to inhibit activated collagenase. Our results are in agreement with the work already has been done by Stricklin and Hoidal, indicating TIMP degradation

with hypochlorite ion.[29] HOCl released from neutrophils rapidly reacts with readily available amines and is thus unable to diffuse away from the immediate pericellular environment of the neutrophil. The most abundant amine in the pericellular environment of the neutrophil is taurine, thus most HOCl is converted to NCT.[9,11–13] We have shown that NCT does not inactivate hrTIMP-1. Thus, oxidative inactivation of hrTIMP-1 is likely to occur only in the pericellular environment of the neutrophil, or in a cellular micro environment, where HOCl is found.

We have also shown that a spectrum of antirheumatic agents has the ability, at sufficient concentrations, to prevent the oxidative inactivation of TIMP-1. In several this occurs at concentrations above serum concentration, however this effect may be relevant in an extracellular microenvironment. For D-penicillamine it is evident at concentrations approaching those reported in patients taking this agent 20 μg/ml (100 μM).[30,31] With the other agents studied however, suppression of HOCl induced inactivation of hrTIMP-1 occurs at concentrations significantly higher than plasma concentrations in patients. However, this in itself does not dismiss a potential therapeutic effect since selective cellular uptake of drugs may result in intracellular concentrations higher than plasma levels. For example, *in vivo* uptake of gold occurs over days or weeks,[32,33] and the final distribution of gold in cells may be very different from simple serum levels. In a study by Wasil and his group, where HOCl inactivated alpha 1-antiprotease, most anti-inflammatory drugs were capable of reacting with HOCl. They have also suggested that, the anti-inflammatory effects of D-penicillamine and gold sodium thiomalate might be the result of rapid scavenging of HOCl by these drugs.[34]

The inactivation of protease inhibitors by oxidants leading to enhanced proteolytic activity has been suggested as a model for neutrophil mediated matrix degradation.[9] Our study enhances this model by demonstrating that it may also apply to TIMP-1. Furthermore we showed that therapeutic agents may interfere with this balance between oxidants and enzyme inhibitors.

### *Acknowledgements*

The authors would like to thank Celltech Pty. Ltd. for their kind gift of recombinant human TIMP-1. We would also like to thank professor Chris Easton for his help and Dr. Dale Halliday for reviewing the manuscript, and Mrs Ludmilla Berkis for technical help.

### *References*

[1] Bigg, H. F., Clark, I. M. and Cawston, T. E. (1994). Fragments of human fibroblast collagenase: interaction with metalloproteinase inhibitors and substrates. *Biochimica et Biophysica Acta*, **1208**, 157–165.

[2] Murphy, G., Docherty, A. J. P., Hembry, R. M. and Reynolds, J. J. (1991). Metalloproteinases and tissue damage. *British Journal of Rhumatology*, **30** (suppl 1), 25–31.

[3] Mallya, S. K., Mookhiar, K. A., Gao, Y., Brew, K., Diozegi, M., Birkedal-Hansen, H. and Van Wart, H. E. (1990). Characterization of 58-Kilodalton human neutrophile collagenase: Comparison with human fibroblast collagenase. *Biochemistry*, **29**, 10628–10634.

[4] O'shea, M., Willenbrock, F., Williamson, R. A., Cockett, M. I., Freedman, R. B., Reynolds, J. J., Docherty, A. J. P. and Murphy, G. (1992). Site directed mutations that alter the inhibitory activity of the tissue inhibitor of metalloproteinases: Importance of the N-terminal region between cysteine 3 and cystein 13. *Biochemistry*, **31**, 10146–10152.

[5] Williamson, R. A., Bryan, J. S., Angal, S., Murphy, G. and Freedman, R. B. (1993). Structural analysis of tissue inhibitor of metalloproteinases-1 (TIMP-1) by tryptic peptide mapping. *Biochimica et Biophysica Acta*, **1164**, 8–16.

[6] Denhardt, D. T., Feng, B., Dylan, R. E., Cocuzzi, E. T. and Malayankar, U. M. (1993). Tissue inhibitor of metalloproteinases (TIMP, aka EPA): Structure, control of expression and biological functions. *Pharmacology and Therapeutics*, **59**, 329–341.

[7] Lohmander, L. S., Hoerrner, L. A. and Lark, M. W. (1993). Metalloproteinases, tissue inhibitor, and proteoglycan fragments in knee synovial fluid in human osteoarthritis. *Arthritis and Rheumatism*, **36**, 181–9.

[8] Birkedal-Hansen, H., Moore, W. G. I., Bodden, M. K., Windsor, L. J., Birkedal-Hansen, B., DeCarlo, A. and Engler, J. (1993). Matrix metalloproteinases: A Review. *Critical Reviews in Oral Biology and Medicine*, **4**, 197–250.

[9] Weiss, S. J. (1989). Mechanisms of disease: Tissue destruction by neutrophils. *The New England Journal of Medicine*, **320**, 365–376.

[10] Laurindo Ieda, M. M., Mello suzana, B. V. and Cossermelli, W. (1995). Influence of low doses of

methotrexate on superoxide anion production by polymorphonuclear leukocytes from patients with rheumatoid arthritis. *Journal of Rheumatology*, **22**, 633–8.
[11] Weiss, S. J. and Peppin, G. J. (1986). Collagenolytic metalloenzymes of the human neutrophil. *Biochemical Pharmacology*, **35**, 3189–3197.
[12] Weiss, S. J., Klein, R., Slivka, A. and Wei, M. (1982). Chlorination of taurine by human neutrophils. *Journal of Clinical Investigation*, **70**, 598–607.
[13] Zgliczynski, J. M., Stelmaszynska, T., Domanski, J. and Ostrowski, W. (1971). Chloramine as intermediates of oxidation reaction of amino acids by myeloperoxidase. *Biochimica et Biophysica Acta*, **235**, 419–424.
[14] Grisham, M. B., Jefferson, M. M., Melton, D. F. and Thomas, E. L. (1984). Chlorination of endogenous amines by isolated neutrophiles. *Journal of Biological Chemistry*, **259**, 10404–10412.
[15] Okada, Y., Watanabe, S., Nakanishi, I., Kishi, J. I., Hayakawa, T., Watorek, W., Travis, J. and Nagase, H. (1988). Inactivation of tissue inhibitor of metalloproteinases by neutrophil elastase and other serine proteinases. *FEBS Letters*, **229**, 157–160.
[16] Davies, J. M. S., Horwits, D. A. and Davies, K. J. A. (1993). Potential roles of hypochlorous acid and N-chloroamines in collagen breakdown by phagocytic cells in synovitis. *Free Radical Biology & Medicine*, **15**, 637–643.
[17] Matheson, N. R., Wong, P. S., Schuyler, M. and Travis, J. (1981). Interaction of human $\alpha_1$-proteinase inhibitor with neutrophile meyloperoxidase. *Biochemistry*, **20**, 331–336.
[18] Frears, E. R., Zhang, Z., Blake, D. R., O'Connell, J. P. and Winyard, P. G. (1996). Inactivation of tissue inhibitor of metalloproteinase-1 by peroxynitrite. *FEBS Letters*, **381**, 21–24.
[19] Cuperus, R. A., Muijsers, A. O. and Wever, R. (1985). Antiarthritic drugs containing thiol groups scavenge hypochlorite and inhibit its formation by myeloperoxidase from human leukocytes. *Arthritis and Rheumatism*, **28**, 1228–1233.
[20] Pekoe, G., Van, Dyke, K., Mengoli, H., Peden, D. and English, D. (1982). Comparison of the effects of antioxidant non-stroidal anti inflammatory drugs against myeloper-oxidase and hypochlorous acid luminol-enhanced chemiluminescence. *Agents & Actions*, **12**, 232–238.
[21] Matheson, N. R. (1982). The effect of anti-arthritic drugs and related compounds on the human neutrophil myeloperoxidase system. *Biochemical and Biophysical Research Communications*, **108**, 159–165.
[22] Bannwart, B., Labat, L., Moride, Y. and Schaeverbck, T. (1994). Methotrexate in rheumatoid arthritis. *Drugs*, **47**, 25–50.
[23] Stevenson, G. A., Lyons, J. G., Cameron, D. A. and O'Grady, R. L. (1985). Rat mammary carcinoma cells in long-term serum free culture provide a continuing supply of collagenase. *Bioscience Report*, **5**, 1071–1077.
[24] Netzel-Arnett, S., Mallya, S. K., Nagase, H., Birkedal-Hanson, H. and Van Wart, H. E. (1991). Continusly recording fluorescent assays optimized for five human matrix metalloproteinases. *Analytical Biochemistry*, **195**, 86–92.
[25] Test, S. T. and Weiss, S. J. (1986). The generation of utilization of chlorinated oxidants by human neutrophils. *Free Radical Biology and Medicine*, **2**, 91–116.
[26] Lark, M. W., Saphos, C. A., Walakovitz, L. A. and Moore, V. L. (1990). In vivo activity of human recombinant tissue inhibitor of metalloproteinases (TIMP). *Biochemical Pharmacology*, **39**, 2041–2049.
[27] Pace, C. N., Shirly, B. A. and Thompson, J. A. Protein structure: a practical approach (Creighton, T E., ed.), PP.311–339 IRL Press, Oxford University Press, Oxford.
[28] Williamson, R. A., Smith, B. J., Angal, S. and Freedman, R. B. (1993). Chemical modification of tissue inhibitor of metalloproteinases-1 and its inactivation by diethyl pyrocarbonate. *Biochimica et Biophysica Acta*, **1203**, 147–154.
[29] Stricklin, J. P. and Hoidal, J. R. (1992). Oxidant-mediated inactivation of TIMP. *Matrix Supplement*, **1**, 325.
[30] Muijsers, A. O., van de Stadt, R. J., Henrichs, A. M. A. and van de Korst, J. K. (1979). Determination of D-penicillamine in serum and urine of patients with rheumatoid arthritis. *Clinica Chimica Acta*, **94**, 173–180.
[31] Van de Stadt, R. J., Muijsers, A. O., Henrichs, A. M. A., van de Korst, J. K. (1979). D-penicillamine: biochemical, metabolic, and pharmacological aspects. *Scandinavian Journal of Rheumatology* (Suppl) **29**, 13–20.
[32] Hurst, N. P., Georjatschko, L., Betts, W. H., Zalewski, P. D. and Forbes, I. J. (1989). Auranofin modulates human neutrophile superoxide production and protein phosphorylation. *Rheumatology International*, **8**, 245–250.
[33] Jellum, E. and Munthe, E. (1982). Fate of the thiomalate part after intramuscular administration of aurothiomalate in rheumatoid arthritis. *Annals of Rheumatic Disease*, **41**, 431–432.
[34] Wasil, M., Halliwell, B., Moorehouse, C. P., Hutchison, D. C. and Baum, H. (1987). Biologically-significant scavenging of the myeloperoxidase-derived oxidant hypochlorous acid by some anti-inflammatory drugs. *Biochemical Pharmacology*, **36(22)**, 3847–3850.