Biochemical Pharmacology, Vol. 52, pp. 105–111, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00168-2

The Gelatinase Inhibitory Activity of Tetracyclines and Chemically Modified Tetracycline Analogues as Measured by a Novel Microtiter Assay for Inhibitors

Liesbet Paemen, Erik Martens,* Koen Norga,* Stefan Masure,* Eugene Roets,† Jos Hoogmartens† and Ghislain Opdenakker*‡*

*Department of Microbiology and Immunology, Laboratory of Molecular Immunology, Rega Institute, and †Institute for Pharmaceutical Research, University of Leuven, Minderbroedersstraat 10, 3000 Leuven, Belgium

ABSTRACT. A quantitative nonisotopic solution assay for gelatinases and inhibitors was developed using biotinylated gelatin as enzyme substrate. In this assay, residual biotinylated substrate is sandwiched between avidin-coated plates and streptavidin-peroxidase and is quantified by the peroxidase reaction. This assay was useful for measuring gelatinase activities and defining the activities of gelatinase inhibitors. When 23 tetracycline analogues were compared, significant differences in gelatinase B inhibition were found between various compounds. 4-epioxytetracycline base, 4-epichlortetracycline, meclocyclinesulfosalicylate, and unmodified metacycline and minocycline proved to be the most potent gelatinase B (EC 3.4.24.35) inhibitors. The gelatinase B inhibitory activity of tetracyclines was clearly dissociated from their antimicrobial activity. The effect of high-molecular-weight inhibitors, such as monoclonal antibodies, was also demonstrable in the microtiter plate assay. In view of the pathophysiological function of gelatinases, the definition of gelatinase inhibitors with known efficacy, safety, and side effects is crucial for the treatment of diseases such as rheumatoid arthritis and multiple sclerosis. Particular tetracyclines fulfil these criteria and the described assay is useful for defining other gelatinase-inhibiting lead compounds. BIOCHEM PHARMACOL 52;1:105–111

KEY WORDS. tetracycline; gelatinase; inhibitor; assay; analogues

TCs§ are widely used therapeutically as antimicrobial agents in many areas of infectious diseases. However, TCs also have important nonantimicrobial properties, that can influence the host response. Thus, TCs have been shown to inhibit the activity of the MMPs collagenase and gelatinase, both *in vivo* and *in vitro* [1, 2]. The latter enzymes play a major role in extracellular matrix remodelling and are found in elevated amounts in the body fluids of patients with tissue-destructive diseases, such as arthritis [3, 4], periodontitis [5], multiple sclerosis [6, 7], and others [8]. The mechanism by which TCs affect MMP activity is not yet known. It has been suggested that the inhibition is linked to the ability of TCs to bind divalent ions, such as Ca^{2+} and to interact with the catalytic Zn. Both mechanisms are essential for the activity of collagenase and gelatinase [9]. Other evidence suggests that TCs can interact with cellular processes such as migration, degranulation, and the synthesis of oxygen radicals [10]. The MMP-inhibitory action of TCs has become of such interest that TC derivatives that have lost their antimicrobial properties altogether, but retain their antiMMP activities are being developed [11, 12]. These are of potential use in the aforementioned chronic inflammatory diseases.

In contrast to collagenases, for which quantitative chromogenic substrate release and substrate conversion assays exist, gelatinases are commonly quantified by zymographic procedures. In the zymographic assay, gelatinases are dissociated from their natural inhibitors and are activated. This impairs the quantification of the net activities of the enzyme and enzyme inhibitor in biological and other samples. Previously, quantitative zymography was used to measure gelatinases in body and tissue culture fluids [3, 6, 7, 13, 14]. Natural inhibitors of metalloproteinases cannot be measured by direct zymography. Indirect zymography-based inhibitor assays can only be used for small diffusable molecules, and require a substantial amount of inhibitor. Finally, reverse zymography as an assay for enzyme inhibitors is labor-intensive. Therefore, a useful quantitative solution assay for gelatinases and gelatinase inhibitors was devel-

‡ Corresponding author. Tel. 32 16 33 73 41; FAX 32 16 33 73 40.

§ *Abbreviations:* APMA, aminophenylmercuric acetate ($H_2NPhAcHg$); CMTC(s), chemically modified tetracycline(s); mAb, monoclonal antibodies; MMP(s), matrix metalloproteinase(s); TC(s), tetracycline(s); TMB, 3,3′,5,5′-tetramethylbenzidine.

Received 19 June 1995; accepted 20 February 1996.

oped, and the activities of a series of inhibitory tetracyclines were compared.

MATERIALS AND METHODS

Reagents

The tetracyclines and analogues mentioned in Table 1 (except minocycline) were made available by the Laboratory for Pharmaceutical Chemistry (University of Leuven, Belgium). Those indicated by an asterisk are commercially available from Acros Chimica (Beerse, Belgium). Minocycline (Minocin®) was obtained from Lederle (Belgium). The gelatin, avidin, and biotin hydroxysuccinimide ester were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). The streptavidin-peroxidase complex was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, U.S.A.). The peroxidase color reagent 3,3′,5,5′-tetramethylbenzidine (TMB) was purchased from Sigma.

The development of mouse monoclonal antibodies was previously described [15]. Alkaline-phosphatase-conjugated antimouse antibodies were purchased from Dako/Prosan (Glostrup, Denmark).

Gelatinase Microplate Assay

The measurement of gelatinase activity was based on the detection of nondegraded biotinylated gelatin with a streptavidin-peroxidase conjugate. This test is based on the biotin-avidin method described by Sorbi *et al.* [16], but modified to a one-microtiterplate assay. Gelatin (1 mg/mL) was biotinylated with a 15-fold molar excess of biotin-N-hydroxysuccinimide ester. Pefabloc (Boehringer) was added to all buffers to prevent the possible intervention of contaminating serine proteases.

Gelatin-sepharose purified gelatinase B (EC 3.4.24.35) [17] was activated by incubation with 2 mM APMA for 2 hr at 37°C. Modified flat-bottomed 96-well ELISA plates (Maxisorp, Nunc Company, Roskilde, Denmark) were coated overnight with 0.5 μg/mL avidin in distilled water. The plates were, then, blocked with 0.1% casein in TBS (50 mM Tris/HCl, pH 7.4, 154 mM NaCl) for 1 hr at 37°C. Biotinylated gelatin was diluted to 50 μg/mL in TBS-T (TBS + 0.05% Tween-20) containing 2 mM Pefabloc, and 100 μL was added to the avidin-coated wells. After incubation for 1 hr at 37°C, the plate was rinsed with TBS-T. Serial dilutions of tetracyclines were made in incubation buffer (50 mM Tris-HCl pH 7.5, 10 mM $CaCl_2$, 0.02% (W/V) NaN_3, 1% (V/V) Triton X-100, 2 mM Pefabloc). Serial dilutions of gelatinase B, starting with 500 ng/mL, were added to the plate as a standard. Tetracycline samples (60 μL) were incubated with gelatinase B (500 ng/mL, 60 μL). Of this mixture, 100 μL was added to the gelatin-coated plates and incubated overnight at 37°C. The plates were, then, washed with TBS-T, and peroxidase-conjugated-streptavidin (Jackson ImmunoResearch Laboratories, dilution 1:20000) was added to each well and incubated for 1 hr at 37°C. After rinsing the plate, the TMB solution was added (3.3 mg/250 μL DMSO, dissolved in citric acid, 0.2 M Na_2HPO_4, pH 4.3, 0.07 μL 30% H_2O_2 per mL) and the optical density of the peroxidase-converted substrate was read at 450 nm.

Inhibition of Denatured Collagen Degradation by Gelatinase B

Gelatinase B inhibitory activity of the tetracyclines was also tested in a collagen-degradation assay, as previously described [15]. Briefly, tracheal septum cartilage (Sigma) was heat-denatured and digested with partially purified neutrophil gelatinase B [17] in the presence or absence of tetracyclines (500 μg/mL). The remaining substrate was detected by SDS-PAGE and degradation was optically evaluated by comparison with undegraded control substrate.

Antimicrobial Activity of Tetracyclines

The ability of the tetracyclines to inhibit bacterial growth was tested on *Escherichia coli* (ATCC 25922). Bacteria were grown in *Luria Bertani* (LB) medium overnight, and 20 μL of a stock containing 1.2×10^9 bacteria/mL was added to vials with 2 mL fresh medium. Tetracyclines, dissolved in LB medium, were added to obtain final concentrations ranging from 0.125 μg/mL to 5 μg/mL. The cultures were incubated overnight at 37°C and optical density of the cultures was read at 600 nm. The O.D. values ranged from 0.0 (medium alone) to 1.6 (medium + bacteria, without tetracycline). The minimal inhibitory concentration (MIC) of the tetracyclines was determined as the concentration at which the O.D. of the culture to which it was added fell below 0.025.

Zymography

Quantitative zymography was performed as described, using 7.5% polyacrylamide gels copolymerised with 0.1% gelatin [13], and enzymatic activity was quantified by computerized image analysis with a PDI scanner (Pharmacia, Belgium) and the PDI software package. For inhibition experiments, minocycline was dissolved in the zymography developing buffer.

RESULTS

Establishment of a Gelatinase Inhibitor Microplate Assay

Gelatin was biotinylated and bound to avidin-coated microtiter plates. By addition of gelatinases, the number of free biotin groups decreased and proportionally less streptavidin-peroxidase was bound to the residual substrate. Bound streptavidin-peroxidase activity was measured spectrophotometrically by the reaction of peroxide with TMB. Inhibition of gelatinase was obtained by the addition of

inhibitor to a known amount of gelatinase prior to application to the plates. The peroxidase activity increased proportionally with the inhibitory activity (Fig. 1).

Plates were first coated with 10 μg/mL avidin and 50 μg/mL biotinylated gelatin (theoretically saturating the avidin binding sites). Because the sensitivity of the assay is dependent on the detection of the minimal amount of degraded biotinylated gelatin, we investigated whether or not decreasing the amount of biotin-gelatin (50 μg/mL-23 ng/mL) bound to the avidin-coated (10 μg/mL) plate increased the sensitivity of the assay. Nonsaturation of the avidin did not enhance sensitivity significantly (data not shown). It was also verified whether or not a decrease in the avidin coating enhanced the sensitivity of the assay (Fig. 2). The optimal avidin concentration for the detection of decreasing gelatinase B activity was determined at 0.3–1.1 μg/mL. In all further experiments, plates were coated with 0.5 μg/mL avidin and 50 μg/mL gelatin-biotin.

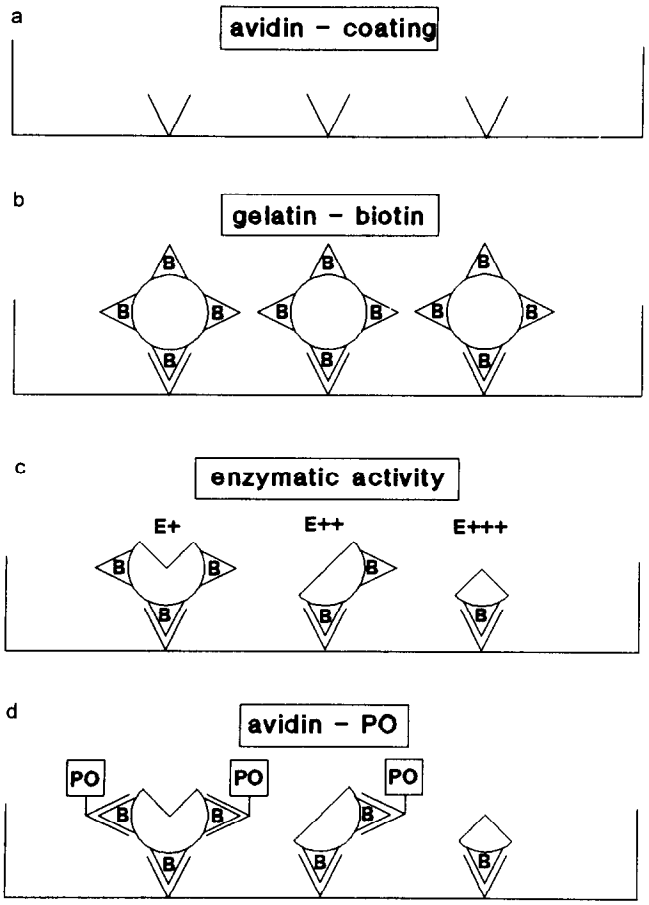


FIG. 1. Flow chart of the gelatinase microtiter plate assay. Individual wells (illustration) of a microtiter plate were coated with avidin (a) to which biotinylated gelatin substrate was bound (b). Enzymatic activity (E) of gelatinases liberated gelatin fragments and decreased the number of residual biotin proportionally (c). The latter was spectrophotometrically quantified by binding of streptavidin-peroxidase and development of peroxidase activity (PO), using peroxide and tetramethylbenzidine as a color reagent (d).

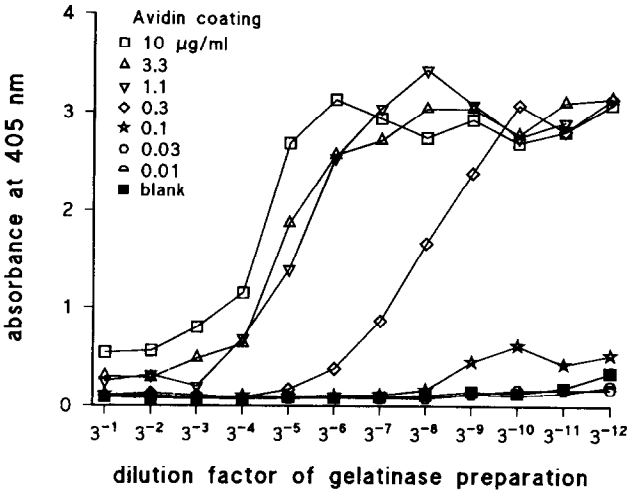


FIG. 2. Effect of avidin coating on the sensitivity of the gelatinase microplate assay. Plates were coated with decreasing amounts (10 μg–10 ng) or without (blank) avidin and, after blocking, incubated with 50 μg/mL gelatin-biotin. The ability of the assay to detect the effect of decreasing amounts of a gelatinase B standard preparation on the degradation of gelatin was measured by streptavidin-peroxidase binding to residual biotinylated gelatin and development of peroxidase activity. The standard preparation contained an equivalent of 60 μg/mL gelatinase activity.

Quantification of Inhibitory Activity

Maximal gelatinase activity (minimal O.D.) was subtracted from lower gelatinase B activities (higher O.D.) to obtain the differential O.D. (ΔO.D.). Decreasing gelatinase B concentrations were inverted to % inhibition against the maximum value (500 ng/mL). ΔO.D. was plotted against % inhibition and used to evaluate the inhibitory activity in the samples (Fig. 3). Although there was some variation in the absolute O.D. values measured in the different assays, the % inhibition calculated for the samples stayed within a 15% margin (n = 4).

Gelatinase B Inhibition of Tetracycline Analogues

Using the classical zymography technique, it was found that minocycline inhibited gelatinase B in a dose-dependent way (Fig. 4). This incited us to test a range of tetracycline analogues for their gelatinase inhibitory capacity. In the microtiter plate assay, change in O.D. between control- and tetracycline-containing wells indicated gelatinase inhibition. Inhibitory potential of the tetracyclines at concentrations of 500 μg/mL, 125 μg/mL, and 31 μg/mL is represented as percent inhibition in Table 1. The validity of the microtiterplate assay was proven by comparison with a collagen-degradation assay [15] (Fig. 5). The results of the CMTCs in the collagen degradation assay are also given in Table 1.

In both assays, 1,10-phenantroline (Sigma) and an inhibitory monoclonal antibody against gelatinase B, REGA-

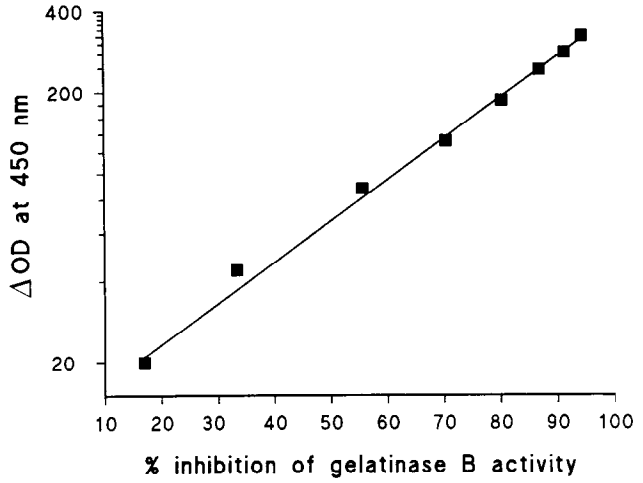


FIG. 3. Percent inhibition plotted against ΔO.D. Maximal gelatinase activity (minimal O.D.) was subtracted from lower gelatinase B activities (higher O.D.) to obtain ΔO.D. Decreasing gelatinase B concentrations were inverted to % inhibition against the maximum value (500 ng/mL), and this relationship was used to evaluate the decreasing activity in the samples. The error range in 4 independent assays was less than 15%.

3G12 [15], were run as positive controls and displayed inhibitory activity (Fig. 5, Table 1).

The different CMTCs displayed variable gelatinase B inhibitory potential. The strongest inhibitors were 4-epioxytetracycline base, 4-epichlortetracycline, meclocyclinesulfosalicylate, and unmodified metacycline and minocycline. Figure 6 illustrates the chemical structure of the CTs and CMTCs (except meclocyclinesulfosalicylate). In general, the 4-epimers of the TCs were more inhibitory. Removal of the methyl group at carbon-6 reduced inhibitory activity as does opening one of the four rings of the tetracycline molecules (e.g., α- and β-apooxytetracycline base, isochlortetracycline).

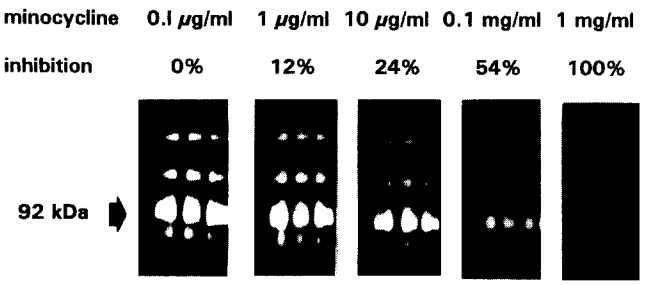


FIG. 4. Inhibition of gelatinase B by minocycline on zymography. A sample of gelatinase B from neutrophils was repetitiously loaded in the slots of a zymography gelatin substrate gel. Slices of the gel were incubated in test tubes in developing buffer without and with increasing amounts of gelatinase B inhibitor. Enzyme activity appeared as colorless bands on a blue background and was quantified by scanning densitometry. Percent inhibition is related to a control to which no minocyline was added. The 92 kDa neutrophil gelatinase monomer is indicated with an arrowhead. Higher molecular mass bands correspond to the gelatinase B dimer and the gelatinase B NGAL heterodimer.

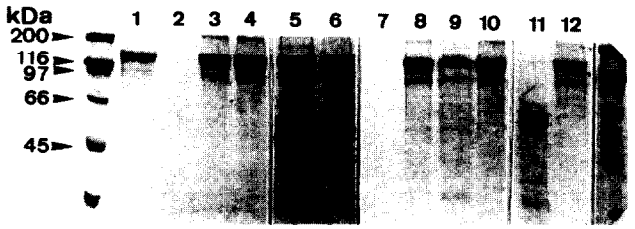


FIG. 5. Inhibition of neutrophil gelatinase B degradation of collagen. Degradation of denatured collagen as monitored by SDS/PAGE. Lane 1, substrate incubated without enzyme; lane 2, substrate incubated with gelatinase B, lanes 3 and 4, substrate incubated with gelatinase B in the presence of 10 and 5 mM 1,10-phenantroline, respectively; lane 5, substrate incubated with gelatinase B in the presence of 30 μg/mL mAb REGA-3G12; lanes 6–12, substrate incubated with gelatinase B in the presence of different CMTCs (500 μg/mL): 4-epianhydrotetracycline, oxytetracycline base, metacycline HCl, anhydrochlortetracycline HCl, minocycline, 4-epidemeclocycline HCl, chlortetracycline, respectively.

Comparison of Gelatinase Inhibitory Activity and Antimicrobial Potency

Because antimicrobial potency was only available for some of the tetracycline analogs we tested [18], we undertook a screening of these molecules using an *Escherichia coli* growth-inhibition test. We concede that antibiotic potency may vary from strain to strain, but present the results of this assay as indicative of antibiotic activity (Table 1).

In general, we found all the 4-epimers, anhydrous, and apoxy-forms to be less antibiotically active than their unmodified counterparts. Thus, for the 4-epimers, antibiotic and antigelatinase B activity are clearly dissociated. Also, tetracycline HCl, oxytetracycline base, demethylchlortetracycline, and demethyltetracycline appear to be strong antibiotics with little or no gelatinase B inhibitory potential in our assays.

DISCUSSION

In view of the disease-promoting role of metalloproteinases in chronic inflammation [19] and cancer [20–24], potent metalloproteinase inhibitors might become important drugs for treatment of these diseases. We established a microtiter plate assay for gelatinolytic activity based on the degradation of biotinylated gelatin. The classical zymography technique is a sensitive enzyme assay that, in addition, yields information about the molecular forms of the gelatinolytic enzymes [13, 14, 17]. For analysis of enzyme inhibitors, however, this novel assay has obvious advantages: the microtiter plate assay is less labor-intensive and, although a larger amount of semi-purified enzyme is necessary, can be performed with at least 1000-fold less inhibitor. Also, inhibitors of all kinds can be evaluated, independent of their molecular weight. In contrast to methods based on the degradation of radiolabeled gelatin, it has the obvious advantage of not requiring a radioisotope safety environment.

TABLE 1. Percent inhibition of gelatinase B activity of the CMTCs as determined by a novel microtiterplate assay (I) and a collagen-degradation assay (II)

	I Microtiter-plate assay CMTC, μg/mL (% inhibition)			II Collagen degradation (CMTC at 500 μg/mL)	III Antibiotic activity, MIC (μg/mL)
	500	125	31		
1. tetracycline HCl*	–	–	–	–	0.6
2. 4-epitetracycline HCl*	20	–	–	–	–
3. anhydrotetracycline HCl*	–	–	–	–	–
4. 4-epianhydrotetracycline HCl*	100	24	–	+	5.0
5. oxytetracycline base*	–	–	–	–	0.6
6. 4-epioxytetracycline base*	100	95	82	+	–
7. α-apooxytetracycline base*	–	–	–	–	–
8. β-apooxytetracycline base*	–	–	–	–	–
9. doxycycline hyclate	90	70	33	+	1.2
10. 6-epidemclocycline HCl*	68	56	11	+	–
11. metacycline HCl	100	95	35	+	1.2
12. demethylchlortetracycline HCl	30	–	–	–	0.6
13. 4-epidemeclocycline HCl	40	15	–	–	–
14. demethyltetracycline base	–	–	–	–	1.2
15. chlortetracycline HCl*	80	35	–	+	2.5
16. 4-epichlortetracycline HCl*	100	100	85	+	5.0
17. isochlortetracycline HCl	–	–	–	–	–
18. anhydrochlortetracycline HCl*	60	20	–	+	5.0
19. 4-epianhydrochlortetracycline HCl*	80	50	30	+	–
20. minocycline HCl	100	70	30	+	2.5
21. 4-epiminocycline HCl	90	80	50	+	2.5
22. 7-aminominocycline HCl	–	–	–	NT	2.5
23. 6-deoxy-6-demethyltetracycline HCl	70	40	–	NT	2.5
24. meclocyclinesulfosalicylaat	100	95	70	+	0.6
1-10-phenantroline	100	100	100	+	NT
	50 μg/mL	25 μg/mL	12 μg/mL		
mAb REGA-3G12	60	10	–	+	NT

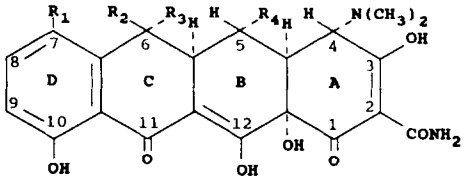
In both assays, 1,10-phenantroline and mAb REGA-3G12 were used as control. In the collagen-degradation assay, – denotes no inhibitory activity of the CMTC at 500 μg/mL, whereas + indicates inhibition, Minimal inhibitory concentration (MIC) of the CMTCs towards *Escherichia coli* is given in III (– indicates no antibiotic activity at 5 μg/mL). Asterisks indicate commercially available TCs. NT, not tested.

Using this microtiter plate assay, we compared the inhibitory effect of a series of tetracycline analogues and gelatinase B-specific antibodies.

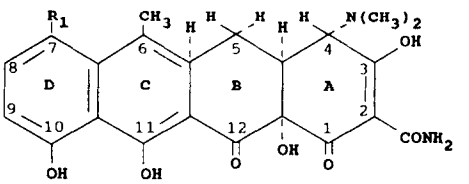
There is increasing support for the potential use of tetracyclines as collagenase inhibitors, rather than antimicrobial agents. The mechanism of collagenase inhibition, however, remains unclear. Based on the inhibition of collagenase degradation of radiolabeled collagen by various CMTCs, it was demonstrated that the carbonyl oxygen and hydroxyl groups of carbon-11 and carbon-12, respectively, are responsible for the anticollagenase activity [25]. This corresponds to an important cation-binding site on the tetracycline molecule, suggesting that inhibition of collagenases occurs through a chelating effect. Our experiments with different CMTCs do not contradict this, but suggest that, at least for the inhibition of gelatinase B, other modifications may also be important. For instance, 4-epimerisation in most cases (minocycline being the exception) increases the antigelatinase B activity of the molecules. Although structural similarities between the members of the MMP family would suggest similarities in the biochemical inhibition mechanism, our results suggest that the inhibitory effect of tetracyclines is MMP-specific; This might have important therapeutic implications.

The alternative use of tetracyclines as proteinase inhibitors has been investigated most extensively in periodontal disease therapy [26–31]. Although tetracyclines were originally applied as antibiotics to reduce putative gingival microflora, it was found that they also help reduce bone resorption [12, 19, 28]. The limited antimicrobial efficacy of TCs, as well as their relative toxicity, has caused them to be replaced by safer and more potent antibiotics. However, clinical trials with low doses of commercially available doxycycline showed a reduction in excessive host collagenase activity in extracts of inflamed gingival tissues, without causing the typical adverse effects of tetracycline treatment [32]. Similarly, a decrease in collagenase activity in synovial tissues and salivary fluid was obtained in clinical trials with low doses of minocycline and doxycycline, respectively, in the treatment of rheumatoid arthritis patients [33]. The use of higher doses, however, in the treatment of more aggressive forms of these diseases may elicit antibiotic side effects, causing the use of classical tetracyclines to be unjustified [34]. For these cases, CMTCs modified to lose

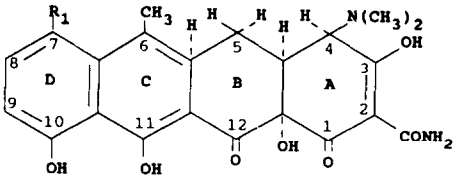
Paemen et al. Figure 6



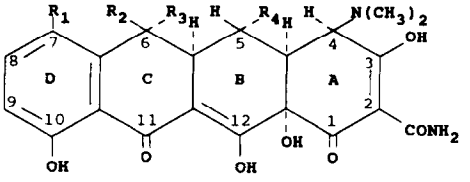
	R_1	R_2	R_3	R_4
Tetracycline (TC)	H	OH	CH_3	H
Oxytetracycline (OTC)	H	OH	CH_3	OH
Doxycycline (DOX)	H	H	CH_3	OH
Chlortetracycline (CTC)	Cl	OH	CH_3	H
Demeclocycline (DMCTC)	Cl	OH	H	H
Metacycline (MTC)	H	-	$=CH_2$	OH
Minocycline (MC)	$-N(CH_3)_2$	H	H	H
6-Epidoxycycline	H	CH_3	H	OH
Demethyltetracycline	H	OH	H	H
6-Deoxy-6-demethyl-tetracycline	H	H	H	H
7-Aminominocycline	NH_2	H	H	H

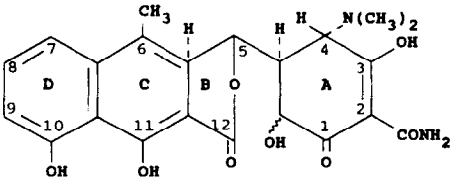


	R1
Anhydrotetracycline (ATC)	H
Anhydrochlor-tetracycline (ACTC)	Cl



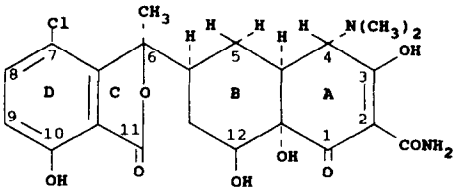
	R1
4-Epianhydrotetracycline (EATC)	H
4-Epianhydrochlor-tetracycline (EACTC)	Cl





α- and β-Apooxytetracycline (α-APOTC, β-APOTC)

	R_1	R_2	R_3	R_4
4-Epitetracycline (ETC)	H	OH	CH_3	H
4-Epioxytetracycline (EOTC)	H	OH	CH_3	OH
4-Epidoxycycline (EDOX)	H	H	CH_3	OH
4-Epichlortetracycline (ECTC)	Cl	OH	CH_3	H
4-Epidemeclocycline (EDMCTC)	Cl	OH	H	H
4-Epiminocycline (EMC)	$-N(CH_3)_2$	H	H	H



Isochlortetracycline (ISOCTC)

FIG. 6. Chemical structure of tetracyclines and tetracycline analogues. All except 4-epidoxycycline were tested in the gelatinase inhibitor microtiter assay.

their antibiotic activity but retain their proteinase inhibitory activity may prove to be beneficial.

The antimetalloproteinase activity of tetracyclines has been found to be effective mainly against collagenases of inflammatory (neutrophil) origin, but not against fibroblast collagenase [26]. This could be a double advantage for their therapeutic use. It would provide a potent inhibitor of neutrophil damage, relatively resistant to other metalloproteinase inhibitors such as α_2-macroglobulin. Additionally, its specificity towards neutrophil collagenases would decrease the risk of TC therapy interfering with normal tissue remodelling and repair [18].

The present study was supported by the National Fund for Scientific Research "Levenslijn—Multiple Sclerose" (NFWO), The General Savings and Retirement Fund (ASLK), the Charcot Foundation, Belgium and the "Vereniging voor Kankerbestrijding." The authors thank Ann Janssen and Ilse Van Aelst for excellent technical assistance and Prof. J. Van Damme, Prof. H. Carton, and Prof. A. Billiau for support. L.P. is an MS research fellow of the NFWO. S.M. is a Cancer Foundation research fellow of the ASLK.

References

1. Arsenis C, Moak SA and Greenwald RA, Tetracylines (TETs) inhibit the synthesis and/or activity of cartilage proteinases in vivo and in vitro. *Matrix-Suppl* **1:** 314, 1992.
2. Golub LM, Greenwald R, Ramamurthy N, Zucker S, Ramsammy L and McNamara T, Tetracyclines (TCs) inhibit matrix metalloproteinases (MMPs): in vivo effects in arthritic and diabetic rats and new in vitro studies. *Matrix-Suppl* **1:** 315–316, 1992.
3. Opdenakker G, Masure S, Grillet B and Van Damme J, Cytokine-mediated regulation of human leukocyte gelatinases and role in arthritis. *Lymphokine Cytokine Res* **10:** 317–324, 1991.
4. Hirose T, Reife RA, Smith GN, Stevens RM, Mainardi CL and Hasty KA, Characterization of type IV collagenase (gelatinase) in synovial fluid of patients with inflammatory arthritis. *J Rheumatol* **19:** 593–599, 1992.
5. Kinane DF, Metalloproteinases in the pathogenesis of periodontal disease. *Curr Opin Dent* **2:** 25–32, 1992.
6. Gijbels KS, Masure S, Carton H and Opdenakker G, Gelatinase in the cerebrospinal fluid of patients with multiple sclerosis and other inflammatory neurological disorders. *J Neuroimmunol* **41:** 29–34, 1992.
7. Paemen L, Olsson T, Söderstrom M, Van Damme J and Opdenakker G, Evaluation of gelatinases and IL-6 in the cerebrospinal fluid of patients with optic neuritis, multiple sclerosis and other inflammatory neurological diseases. *Eur J Neurol* **1:** 55–63, 1994.
8. Woessner FJ, Matrix metalloproteinases and their inhibitors in connective tissue remodeling. *FASEB J* **5:** 2145–2154, 1991.
9. Vallee B and Auld D, Zinc coordination, function, and structure of zinc enzymes and other proteins. *Biochemistry* **29:** 5647–5659, 1990.
10. Gabler WL and Creamer HR, Suppression of human neutrophil functions by tetracyclines. *J Periodont Res* **26:** 52–58, 1991.
11. Golub LM, Suomalainen K and Sorsa T, Host modulation with tetracyclines and their chemically modified analogs. *Curr Opin Dent* **2:** 80–90, 1992.
12. Rifkin BR, Vernillo AT and Golub LM, Blocking periodontal disease progression by inhibiting tissue destructive enzymes: a potential therapeutic role for tetracylines and their chemically modified analogs. *J Periodontol* **64:** 819–827, 1994.
13. Masure S, Billiau F, Van Damme J and Opdenakker G, Human hepatoma cells produce an 85 kDa gelatinase regulated by phorbol 12-myristate 13-acetate. *Biochim Biophys Acta* **1054:** 317–325, 1990.
14. Van Ranst M, Norga K, Masure S, Proost P, Vandekerckhove P, Auwerx J, Van Damme J and Opdenakker G, The cytokine-protease connection: identification of a 96-kD THP-1 gelatinase and regulation by interleukin-1 and cytokine inducers. *Cytokine* **3:** 231–236, 1991.
15. Paemen L, Martens E, Masure S and Opdenakker G, Monoclonal antibodies specific for natural human neutrophil gelatinase B used for affinity purification, quantitation by two-site ELISA and inhibition of enzymatic activity. *Eur J Biochem* **234:** 759–765, 1995.
16. Sorbi D, Fadly M, Hicks R, Alexander S and Arbeit L, Captopril inhibits the 72 kDa and 92 kDa matrix metalloproteinases. *Kidney Intern* **44:** 1266–1272, 1993.
17. Masure S, Proost P, Van Damme J and Opdenakker G, Purification of 91-kDa neutrophil gelatinase. Release by the activating peptide interleukin-8. *Eur J Biochem* **198:** 391–398, 1991.
18. Mitscher L, The chemistry of the tetracycline antibiotics. Marcel Dekker, Inc., New York, 1978.
19. Golub LM, Ramamurthy NS, McNamara TF, Greenwald RA and Rifkin BR, Tetracyclines inhibit connective tissue breakdown: new therapeutic implications for an old family of drugs. *Crit Rev Oral Biol Med* **2:** 297–321, 1991.
20. Opdenakker G and Van Damme J, Cytokine-regulated proteases in autoimmune diseases. *Immunol Today* **15:** 103–107, 1994.
21. Opdenakker G and Van Damme J, Cytokines and proteases in invasive processes: molecular similarities between inflammation and cancer. *Cytokine* **4:** 251–258, 1992.
22. Vassalli JD and Pepper M, Membrane proteases in focus. *Nature* **370:** 14–15, 1994.
23. Sato H, Takino T, Okada Y, Cao J, Shinagawa A, Yamamoto E and Seiki M, A matrix metalloproteinases expressed on the surface of invasive tumour cells. *Nature* **370:** 61–64, 1994.
24. Golub L, Lee M, Lehrer H, Nemiroff G, McNamara A, Kaplan R and Ramamurthy N, Minocycline reduces gingival collagenolytic activity during diabetes: preliminary observations and a proposed new mechanism of action. *J Periodont Res* **18:** 516–526, 1983.
25. Magnam J, Barthes D and Giraud JJ, Tetracycline complexes. Study of 1-1 complexes of iron III, cobalt II, copper II and nickel II with tetracycline, demeclocycline, doxycycline and minocycline. *Ann Pharm Franc* **42:** 155–159, 1984.
26. Golub LM, McNamara TF, D'Angelo G, Greenwald RA and Ramamurthy NS, A non-antimicrobial chemically-modified tetracycline inhibits mamalian collagenase activity. *J Dent Res* **66:** 1310–4, 1987.
27. Lee W, Aitkin S, Kulkarni G, Birek P, Overall CM, Sodek J and McCulloch CA, Collagenase activity in recurrent periodontitis: relationship to disease progression and doxycycline therapy. *J Periodontal Res* **26:** 479–485, 1991.
28. Moskow BS and Tannenbaum P, Enhanced repair and regeneration of periodontal lesions in tetracycline-treated patients. Case reports. *J Periodont* **62:** 341–350, 1991.
29. Suomalainen K, Halinen S, Ingman T, Lindy O, Saari H, Konttinen Y, Golub L and Sorsa T, Tetracycline inhibition identifies the cellular sources of collagenase in gingival crevicular fluid in different forms of periodontal diseases. *Drugs Exp Clin Res* **18:** 99–104, 1992.
30. Golub LM, Ciancio S, Ramamurthy N, Leung M, McNamara TF, Low-dose doxycycline therapy: effect on gingival and crevicular fluid collagenase activity in humans. *J Periodont Res* **25:** 321–330, 1990.
31. Vernillo AT, Ramamurthy NS, Golub LM, Rifkin BR, The non-antimicrobial properties of tetracycline for the treatment of periodontal disease. *Curr Opin Periodont* **111:** 8, 1994.
32. McNamara TF, Golub LM, Yu Z, Ramamurthy NS, Reduced doxycycline blood levels in humans fail to promote resistant organisms. In: *International conference on periodontal disease: Pathogens and Host immune responses, 25–27 Sept., 1990*, Osaka University Press, Osaka, Japan.
33. Lauhio A, Konttinen Y, Tschesche H, Nordström D, Salo T, Lähdevirta J, Golub L, Sorsa T, Reduction of matrix metalloproteinase 8-neutrophil collagenase levels during long-term doxycycline treatment of reactive arthritis. *Antimicrob Agents Chemother* **38:** 400–402, 1994.
34. McCullough CA, Birek P, Overall C, Aitken S, Lee W, Kulkarni G, Randomized controlled trial of doxycycline in prevention of recurrent periodontitis in high risk patients: antimicrobial activity and collagenase inhibition. *J Clin Periodontol* **17:** 616–621, 1990.