Dental Research

A Non-antibacterial Chemically-modified Tetracycline Inhibits Mammalian Collagenase Activity

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Tetracyclines (including the semi-synthetic analogues, minocycline and doxycycline) are considered useful adjuncts in periodontal therapy because they suppress Gram-negative periodontopathogens. Recently, these antibiotics were found to inhibit mammalian collagenase activity, a property which may also be of therapeutic value. It has been suggested that the anti-collagenase properties of the tetracyclines are independent of their antibiotic efficacy. To advance this hypothesis further, we chemically converted tetracycline hydrochloride to its non-antimicrobial analogue, de-dimethylaminotetracycline. This chemically-modified tetracycline (CMT), although no longer an effective antibiotic, was found to inhibit the in vitro activity of collagenase from partially purified extracts of human rheumatoid synovial tissue and rachitic rat epiphysis. In a preliminary in vivo study, pathologically-excessive collagenase in skin and gingiva was induced by rendering adult male rats diabetic, and the oral administration of CMT to these rats significantly reduced the excessive collagenase activity in both tissues. Moreover, CMT administration did not affect the severe hyperglycemia in these rats but did prevent, at least in part, the diabetes-induced loss of body weight, skin weight, and skin collagen mass; these effects suggest a lack of toxicity in this animal model. A proposed clinical advantage of CMT over conventional tetracyclines, in the treatment of diseases characterized by excessive collagenolytic activity, is the lack of development of antibiotic-resistant micro-organisms during prolonged use. However, the consideration of clinical trials to support this hypothesis must await further laboratory and extensive toxicity tests.

Introduction.

The therapeutic potential of the tetracycline antibiotics as adjuncts in the treatment of periodontal diseases has traditionally been ascribed to two factors: (i) their ability to suppress micro-organisms such as *Bacteroides gingivalis* and *Actinobacillus actinomycetemcomitans*, which are believed to be periodontopathogens (Slots and Rosling, 1983; Ciancio et al., 1982; Lindhe, 1982; Lindhe et al., 1983; Slots et al., 1979; Kornman and Karl, 1982); and (ii) their concentration at the site of the lesion, within the gingival crevicular fluid, at levels much higher than those found in serum (Gordon et al., 1981; Ciancio et al., 1980). Recently, Golub et al. (1983, 1984, 1985a,b) introduced a new concept concerning the therapeutic usefulness of the tetracyclines. They proposed that: (i) tetracyclines, but not other antibiotics, can inhibit the activity of collagenase (a specific collagenolytic metallo-neutral protease produced by host tissues which has repeatedly been implicated in periodontal destruction; see Birkedal-Hansen, 1980, for review); and (ii) this newly discovered property of the drugs could provide a novel approach to the treatment of diseases, such as periodontal disease, but also including certain medical disorders

(*e.g.*, non-infected corneal ulcers; Perry and Golub, 1985; Perry et al., 1986), which involve excessive collagen destruction.

Tetracycline(s) appear to inhibit collagenase activity by a mechanism unrelated to the drug's antibacterial efficacy (see "Discussion"). In a recent preliminary report, McNamara et al. (1986) described a minimal chemical modification of the tetracycline molecule (*i.e.*, the removal of the dimethylamino group from the C_4 position on the 'A' ring), which eliminated the drug's antibiotic efficacy both *in vitro* and *in vivo*. In the current study, we investigated the ability of this non-antimicrobial chemically-modified tetracycline (CMT) to inhibit collagenase activity. The purpose was to determine, in a manner more direct than previously attempted, whether the anti-collagenase and antibiotic properties of the drug are unrelated, each being associated with a different part of the drug molecule. Of clinical relevance, the long-term use of such an agent would have the potential to inhibit collagen destruction during chronic disease *without* evoking a major antibiotic complication, namely, the emergence of tetracycline-resistant micro-organisms.

Materials and methods.

Tetracycline (Sigma Chemical Co., St. Louis, MO) was chemically modified by removing the dimethylamino group from the C_4 position of the 'A' ring of the molecule, since the normal α-orientation of this group is essential for the drug's antibiotic efficacy (Mitscher, 1978). The procedure was based on previously published techniques (McCormick et al., 1957; Boothe et al., 1958). The details of the synthesis, chemical characterization (including the ultra-violet and infrared spectra and thin-layer chromatography Rf values), and loss of antibacterial activity against *Bacillus cereus* (the organism typically used in standard bioassays for tetracyclines) and against several periodontopathogens (*Fusobacterium nucleatum* and *B. gingivalis*) will be described elsewhere.

In brief, tetracycline was reacted (six days, 22°C) in tetrahydrofuran with iodomethane to produce the intermediate, tetracycline methiodide. The solid tetracycline methiodide was precipitated, collected by vacuum filtration, washed with cold tetrahydrofuran and diethyl ether, and vacuum-dried at 50°C. The methiodide intermediate was found to have a melting point of 178-180°C, in good agreement with the findings of McCormick et al. (1957). The intermediate was then dissolved in 50% acetic acid containing powdered zinc (Zn) (17 mg/mL). The suspension was stirred for 30 minutes, the excess Zn was filtered off, and, after addition of cold (0-5°C) dilute HCl to the filtrate, a yellow precipitate formed. This compound, the chemically-modified tetracycline (CMT) or de-dimethylaminotetracycline, was washed and re-crystallized from an ethyl acetate-petroleum ether (BP 60-90°C) mixture and was found to have a melting point of 195-197°C, in agreement with previously published data (see above).

The CMT was administered by oral intubation on a daily basis to diabetic rats with pathologically-excessive collagenase

activity, by means of techniques described previously (Golub *et al.*, 1983, 1984, 1985b). In the first of two studies, four groups of adult (body weight from 350 to 400 g each) male Sprague-Dawley rats were established as follows: non-diabetic control rats (C group; n = 4 rats), rats in which diabetes was induced by i.v. injection of streptozotocin (D group; n = 3); and two additional groups of diabetic rats which received either metronidazole (70 mg per day; D + met group; n = 5 rats) or CMT (20 mg per day; D + CMT group; n = 4 rats) throughout the experimental protocol. The regimen, 20 mg per day of CMT, was chosen for this initial set of studies because the same dose of minocycline, an antibiotically-active tetracycline, was successfully administered to diabetic rats in previous experiments by Golub *et al.* (1983 & 1985b). Moreover, in humans, metronidazole is administered at a level about 3.8 times greater than the dosage prescribed for minocycline, and this dose, 70 mg metronidazole per day, was found to have no inhibitory effect on rat collagenase activity *in vivo* (Ramamurthy *et al.*, 1986).

Thirty-seven days after induction of diabetes (drug therapy was initiated one day after streptozotocin administration), the rats were weighed (they had previously been weighed at time 0, the day some were injected with streptozotocin, and at several time periods during the experimental protocol), and a blood sample was taken for glucose analysis. The animals were then killed, the entire skin was dissected and weighed, and samples (about 100 mg) of the tissue were analyzed for hydroxyproline (after hydrolysis in 6 mol/L HCl, 105°C, 24 hr) and for collagenase activity (see below) for each rat separately. The buccal gingivae from the maxillary jaws were dissected and pooled per group (individual rats did not yield sufficient gingivae to assay for collagenase activity in the tissue extracts). As described in detail previously (Ramamurthy and Golub, 1983; Golub *et al.*, 1985b), the tissues were minced and extracted, the extracts partially purified by ammonium sulfate precipitation, and the collagenase activity in the extracts was measured with either ^{14}C-gly labeled collagen or [^{3}H-methyl] collagen used as substrate.

The second study in this series was essentially the same as that described above, except that it was carried out for 21 rather than 37 days, and one of the diabetic groups was treated with minocycline (5 mg per day) instead of metronidazole. This lower-than-usual dose of minocycline had previously been found to be effective in a preliminary study (Ramamurthy *et al.*, unpublished data). Each of the four groups in study no. 2 (Controls, Diabetics, D + minocycline, and D + CMT) contained four rats.

To determine whether CMT could directly inhibit a mammalian collagenase *in vitro*, we performed two experiments. First, synovial tissue was obtained from knee joints of five adult humans with severe rheumatoid arthritis who required total joint implant surgery (Greenwald *et al.*, 1986). A 200-mg sample of each specimen was lyophilized, minced with scissors, ground with a mortar and pestle, extracted three times with petroleum ether for removal of lipids, and dried, and the collagenase activity was extracted as described by Ramamurthy and Golub (1983). All of these steps were performed at 4°C. The latent collagenase in the partially-purified extract was activated by brief pre-treatment with 4 μmol/L trypsin (10 min, 22°C), which was then inactivated with a five-fold molar excess of soybean trypsin-inhibitor. The trypsin-activated synovial collagenase was incubated with ^{14}C-gly-labeled collagen fibrils for 24 hr at 35°C (Ramamurthy and Golub, 1983) in the presence of 0, 2, 5, 10, and 20 μg/mL CMT or 0, 5, and 20 μg/mL minocycline. The radiolabeled collagen degradation products were separated from the undigested fibrils and counted in a liquid scintillation spectrometer as described previously.

(Note: Trypsin degraded 7.3% ± 1.9 of the ^{14}C-collagen substrate, confirming the expected resistance of the collagen substrate to non-specific protease attack.) In a second experiment to evaluate the *in vitro* inhibition of mammalian collagenase, the tetracyclines, CMT or minocycline, were added to extracts of rachitic rat epiphyseal cartilage. Induction of rickets by a low-phosphate, vitamin-D-deficient diet results in extremely high collagenase activity in the growth plates of rachitic long bones (Dean *et al.*, 1985). Collagenase activity was measured in duplicate aliquots of rachitic cartilage extract with ^{14}C-gly-labeled collagen fibrils after 48 hours of incubation as described above.

The data were analyzed by analysis of variance, and the significance of the differences between the groups was analyzed by Tukey's test (Snedecor and Cochran, 1967).

Results.

As shown in Table 1, streptozotocin-induced diabetes increased the blood glucose concentration about 5-8-fold, compared with control values (p<0.01), and there was no significant difference (p>0.05) in this parameter between the four groups of diabetics (D, D + metronidazole, D + CMT, D + minocycline).

As expected from previous studies (Golub *et al.*, 1983; Schneir and Golub, 1981), induction of a severely hyperglycemic, diabetic state in the rat produced both a dramatic increase in skin collagenase activity (skin tissue from the control group produced no detectable collagenolytic activity above trypsin activity values) and about a 50-60% loss of skin weight (studies no. 1 and 2; Table 1). These differences between the C and D groups were statistically significant (p<0.01). Treating the diabetic rats with metronidazole produced no significant effect on any of the parameters in skin, including skin weight, collagen content, and collagenase activity (study no. 1). In contrast, CMT treatment reduced the pathologically-excessive collagenase activity by 55% in both studies no. 1 and 2, an effect found to be statistically significant (p<0.01). CMT administration also increased skin weight in the diabetic rats by 60-88% (studies 1 and 2), compared with the values seen in the untreated diabetics (p<0.01). These anti-catabolic effects of CMT on connective tissue metabolism were *not* associated with a significant effect on the severity of the hyperglycemic state (Table 1). The changes in skin collagen mass appeared to follow the same pattern as skin weight. Although these changes in skin hydroxyproline were found to be statistically significant in study no. 2, this was not the case for study no. 1. In previous studies where the same animal model was used, diabetes produced a significant loss of skin collagen (Schneir and Golub, 1981; Golub *et al.*, 1983). The tetracycline antibiotic, minocycline, had essentially the same effect on the skin parameters as did CMT (study no. 2).

Parallel changes were seen in the pools of gingival tissue from four of these groups of rats. The collagenase activities (expressed as the % [^{3}H-methyl]-collagen degraded) in the gingivae from the control, diabetic, diabetic + metronidazole, and diabetic + CMT rats were 0%, 97.8%, 93.9%, and 53.0%, respectively.

As shown in the Fig., the control rats gained weight during the 37-day protocol (about a 19% increase, compared with time 0 values; study no. 1), while both the untreated diabetics and the diabetics treated with metronidazole lost 21% of their original weight. CMT (and minocycline; see study no. 2) treatment prevented the loss of body weight seen in the other two groups of diabetic rats. Similar changes in the control and diabetic groups were seen in study no. 2 and, once again, tetracycline

TABLE 1

STREPTOZOTOCIN-INDUCED DIABETES IN THE RAT STIMULATES COLLAGENASE ACTIVITY IN SKIN: EFFECT OF ORAL
ADMINISTRATION OF CMT, MINOCYCLINE, OR METRONIDAZOLE*

Experimental Group	Blood Glucose (mg/dL)	Skin Weight (g)	Skin Collagen Content (mg Hyp/total skin)	Skin Collagenase Activity: ^{14}C-Collagen lysed (%)
Study No. 1:				
Controls	103 ± 15[A]	63.7 ± 4.7[A,a]	1160 ± 127	5.2 ± 0.9[A]
Diabetics (D)	850 ± 60[B]	30.7 ± 2.3[B]	617 ± 65	47.3 ± 0.3[B]
D + metronidazole	638 ± 50[B]	25.9 ± 3.5[B]	654 ± 95	46.0 ± 3.0[B]
D + CMT	713 ± 62[B]	49.1 ± 3.2[A,b]	850 ± 64	20.9 ± 3.2[C]
Study No. 2:				
Controls	113 ± 6[A]	62.1 ± 3.3[A]	1650 ± 168[A]	9.4 ± 0.3[A]
Diabetics (D)	552 ± 38[B]	27.8 ± 3.3[B]	660 ± 81[B,a]	38.1 ± 2.1[B]
D + minocycline	548 ± 30[B]	43.0 ± 7.2[A]	1130 ± 69[B,b]	19.2 ± 1.0[C]
D + CMT	553 ± 31[B]	52.5 ± 6.8[A]	1040 ± 64[B,b]	17.2 ± 0.1[C]

*Each value represents the mean ± S.E.
[A,B,C] p < 0.01; [a,b,c] p < 0.05. Group values with different symbols (*e.g.*, A *vs.* B.) are significantly different at the levels indicated.
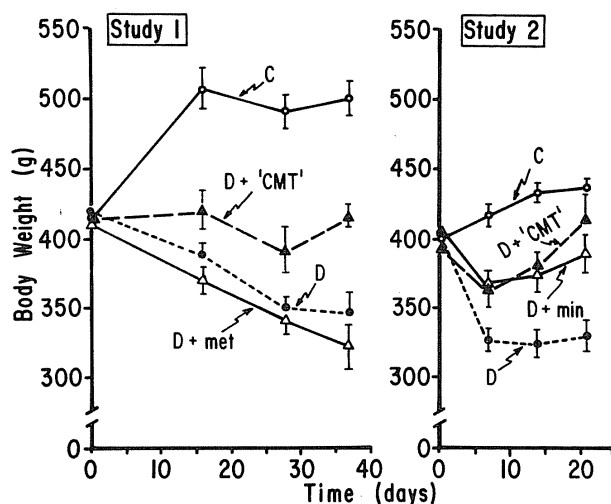

Fig. — Sequential changes in body weight during the 37-day (study no. 1) and 21-day (study no. 2) experimental protocols. C = non-diabetic control rats; D = untreated diabetics; D+met, D+min, D+CMT = diabetics treated on a daily basis with metronidazole, minocycline, and chemically-modified tetracycline, respectively. Each value represents the mean ± S.E.

(this time minocycline as well as CMT) prevented body weight loss.

In the first *in vitro* study, the activated collagenase in the partially purified extracts of human synovial tissue degraded about 23% of the ^{14}C-collagen fibril substrate (Table 2). Adding CMT to the reaction mixture in concentrations ranging from 2-20 μg/mL decreased the collagenase activity by 35-94%, respectively — effects that were statistically significant. At the two concentrations tested (5 and 20 μg/mL), minocycline inhibited collagenase activity to the same extent as did CMT. In the second *in vitro* study, a comparable effect was also noted with the rachitic cartilage extract and these two tetracyclines (Table 3).

Discussion.

Tetracyclines are broad-spectrum antibiotics which, through their ability to inhibit bacterial protein synthesis, can suppress periodontopathic organisms in the oral cavity. Numerous clin-ical studies (see ''Introduction'') as well as case reports (Fasciano and Fazio, 1981; Moskow, 1986) support the usefulness of tetracyclines as adjuncts in the treatment of periodontal diseases. A recent series of studies addressed the additional ability of these drugs to inhibit the activity of mammalian collagenases and the likelihood that this newly discovered property contributes significantly to their clinical efficacy in the management of periodontal and perhaps other diseases as well (Golub *et al.*, 1983, 1984, 1985a,b; Gomes *et al.*, 1984; Zucker *et al.*, 1985; Perry and Golub, 1985; Seedor *et al.*, 1985; Greenwald *et al.*, 1986, 1987).

In the present study, CMT decreased collagenolytic activity in skin and gingivae when administered to diabetic rats *in vivo*, and when incubated with mammalian connective tissue collagenases *in vitro*. We have reported elsewhere that CMT, when added to a reaction mixture containing [^{3}H-methyl] collagen and synovial fluid from a patient with severe rheumatoid arthritis, was found to inhibit both the breakdown and loss of α-collagen components and the generation of α [A]digestion products (assessed by a combination of SDS-polyacrylamide gel electrophoresis and fluorography; Greenwald *et al.*, 1987). Consistent with our previous reports (Ramamurthy *et al.*, 1986), metronidazole (a non-tetracycline antibiotic also used clinically as an adjunct in periodontal therapy; Loesche *et al.*, 1981) had no inhibitory effect on the collagenase activity in rat skin and gingiva, whereas minocycline (an antibiotic tetracycline recently shown to be effective in reducing the severity of periodontal disease; Ciancio *et al.*, 1980 and 1982) seemed to be equally as effective as CMT in inhibiting collagenase activity *in vivo* and *in vitro*.

The mechanism by which tetracyclines inhibit collagenase appears to be independent of their antibiotic activity. The evidence includes the following facts: (i) These drugs inhibit collagenase activity and collagen breakdown in germ-free as well as conventional rats (Golub *et al.*, 1983; Golub *et al.*, 1985b); (ii) their administration *in vivo* inhibits collagenase activity and/or collagen destruction in non-infected tissues of humans (Perry and Golub, 1985; Greenwald *et al.*, 1986), as well as in experimental animals (Golub *et al.*, 1983; Golub *et al.*, 1985b; Seedor *et al.*, 1985); (iii) these drugs directly inhibit mammalian collagenolytic activity *in vitro* (Golub *et al.*, 1983; Golub *et al.*, 1984; Gomes *et al.*, 1984; Golub *et al.*, 1985a,b; Zucker *et al.*, 1985); and (iv) their administration to humans in low doses substantially reduces the collagenase activity in the fluid of the periodontal pocket without producing a detectable effect on the subgingival microflora (Golub *et al.*, 1985a; Golub *et al.*, 1987). The present study provides direct

TABLE 2

A CHEMICALLY-MODIFIED TETRACYCLINE (CMT) INHIBITS COLLAGENASE ACTIVITY FROM HUMAN RHEUMATOID SYNOVIUM *IN VITRO*: A COMPARISON WITH THE INHIBITORY EFFECT OF MINOCYCLINE*

Incubation Conditions	^{14}C-collagen gel lysis (%)		% Inhibition of Collagenase activity by	
	CMT	Minocycline	CMT	Minocycline
1. Synovial collagenase alone (0 μg/mL drug)	22.6 ± 2.2	22.6 ± 2.2	0	0
2. Collagenase + 2 μg/mL drug	15.0 ± 2.2[a]	—	35	—
3. Collagenase + 5 μg/mL drug	10.1 ± 1.6[b]	11.3 ± 2.1[b]	57	52
4. Collagenase + 10 μg/mL drug	4.7	—	78	—
5. Collagenase + 20 μg/mL drug	1.3 ± 0.5[b]	1.0 ± 0.4[b]	94	96

*Each value represents the mean value ±S.E. for five patients with severe rheumatoid arthritis except for incubation no. 4, which is the value for one patient only.

[a]$p< 0.05$, 1 *vs.* 2.

[b]$p<0.01$, 1 *vs.* 3 and 1 *vs.* 5.

TABLE 3

EFFECT OF CMT AND MINOCYCLINE ON COLLAGENASE ACTIVITY IN EXTRACTS OF RACHITIC RAT CARTILAGE

Incubation Conditions	^{14}C-collagen gel lysis (%)*		% Inhibition of Collagenase activity by	
	CMT	Minocycline	CMT	Minocycline
Femoral (FEM) cartilage extract + 0μg/mL drug	40.4	40.4	0	0
Tibial (TIB) cartilage extract + 0μg/mL drug	51.4	51.4	0	0
FEM + 3μg/mL drug	30.3	–	25.0	–
TIB + 3μg/mL drug	32.6	–	36.5	–
FEM + 10μg/mL drug	23.7	–	41.3	–
TIB + 10μg/mL drug	–	19.9	–	61.3
FEM + 20μg/mL drug	8.8	–	78.2	–
TIB + 20μg/mL drug	–	13.7	–	73.3

*Each value represents the mean of duplicate analyses.

evidence that the antibiotic and anti-collagenase properties of tetracycline reside in different parts of the molecule, since chemically modifying the drug to eliminate its anti-bacterial efficacy (Mitscher, 1978; McNamara *et al.*, 1986) did not reduce its anti-collagenase activity. Golub *et al.* (1983) suggested that tetracycline's inhibitory effect on collagenase might relate to the drug's well-known ability to bind metal ions (Ca^{2+} and Zn^{2+}; see Ross and Picozzi, 1985), since this enzyme is dependent on these cations to maintain its normal conformation and hydrolytic activity (Berman, 1980). The current study supports this proposal by demonstrating that the removal of the dimethylamino group at C_4, which eliminates the drug's antibacterial efficacy, does not affect its anti-collagenase properties, presumably because the metal-binding carbonyl and hydroxyl groups, on the opposite side of the tetracycline molecule, are unaffected by the chemical modification (future studies will compare the Ca^{2+} and Zn^{2+} binding capacity of tetracycline and CMT). This proposal is supported by the following: (i) Minocycline (an antibacterial tetracycline which retains the dimethylamino group) and CMT (a non-antibacterial tetracycline lacking this group at C_4) appear to be equally effective as inhibitors of mammalian collagenase *in vivo* and *in vitro* (see Tables 1-3); (ii) consistent with these results, Golub *et al.* (1983, 1985b) reported that the *in vivo* administration of minocycline (20 mg per day) produced about a 60% reduction in the pathologically-excessive collagenase activity in diabetic rat tissues (skin, gingivae). The administration of the same dose of CMT to diabetic rats resulted in a similar reduction of collagenase activity in the same tissues in the current study; and (iii) the role of cations in the anti-collagenase effectiveness of tetracyclines, including chemically-modified analogues, is suggested by the observation that excess Ca^{2+} can overcome the inhibition of the enzyme by the drugs *in vitro* (Golub *et al.*, 1983; Zucker *et al.*, 1985).

The clinical potential of the anti-collagenase properties of tetracyclines is now being considered in the management of a number of disease processes involving collagen catabolism (see Sheridan, 1984). These include the healing of non-infected corneal ulcers (Perry and Golub, 1985; Perry *et al.*, 1986; Seedor *et al.*, 1985), the inhibition of collagenase in the joint tissue and fluid of patients with rheumatoid arthritis (Greenwald *et al.*, 1986, 1987), the reduction of collagenolytic activity generated by melanoma tumor cells (Zucker *et al.*, 1985), the inhibition of excessive bone resorption induced either by systemic (Gomes *et al.*, 1984) or local factors (Golub *et al.*, 1984), and, of course, the reduction of collagenolysis in the soft as well as hard tissues during periodontal disease (Golub *et al.*, 1983, 1984, 1985a,b). Consistent with this approach, Simonson *et al.* (1986) recently demonstrated that minocycline and doxycycline (both tetracyclines), administered *in vivo* to rachitic rats, can suppress epiphyseal cartilage collagenase activity and restore some parameters of abnormal bone metabolism toward normal. However, it is recognized that other steps in the breakdown of connective tissues, in addition to the extracellular activity of collagenase, may be inhibited by tetracyclines. Possibilities include (but are not limited to) the

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