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**INHIBITION OF MATRIX
METALLOPROTEINASES
THERAPEUTIC APPLICATIONS**

*Edited by Robert A. Greenwald, Stanley Zucker,
and Lorne M. Golub*

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The cover of the paper-bound edition of this volume shows the catalytic domain of a crystal structure of truncated MMP-3 bound to an MMP inhibitor. The inhibitor shown is PGE-116611, the chemical name of which is: (2R)-isobutyl-(3S)-[N-hydroxycarboxamido]-6-hydroxyhexanoic acid amide of (1N)-2-[methoxyethyl]-caprolactam-(3S)-amine. The cover illustration was generously provided by Drs. Biswanath De and Glen Mieling of Procter and Gamble Pharmaceuticals.

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Non-antimicrobial and Antimicrobial Tetracyclines Inhibit IL-6 Expression in Murine Osteoblasts

KEITH L. KIRKWOOD,^{a,b,f} LORNE M. GOLUB,^c AND PETER G. BRADFORD^{b,d,e}

^aDepartment of Periodontics and ^bCenter for Molecular Mechanisms of Diseases and Aging, State University of New York at Buffalo, Buffalo, New York 14214, USA

^cDepartment of Oral Biology and Pathology, State University of New York at Stony Brook, Stony Brook, New York 11794, USA

^dDepartment of Pharmacology and Toxicology and ^eDepartment of Oral Biology, State University of New York at Buffalo, Buffalo, New York 14214, USA

IL-6 regulation in osteoblasts has been the focus of several groups, since this cytokine appears to contribute toward the pathophysiological state of postmenopausal osteopenia.¹ IL-6 is the major cytokine regulated by sex steroids as compared to other cytokines, such as IL-1 β , IL-11, and TNF- α , that are not regulated by sex steroids.² Previous results from our laboratory have indicated that intracellular calcium is a critical determinant of the osteoblast secretory capacity.^{3,4} Since tetracycline is well known for its ability to bind divalent cations, such as calcium and zinc, and to affect intracellular calcium concentrations,⁵ we evaluated the abilities of doxycycline and chemically modified tetracyclines, which lack antimicrobial activity, to affect osteoblast IL-6 secretion from MC3T3-E1 osteoblastic cells.

METHODS

IL-6 Secretion Measurements

MC3T3-E1 cells were cultured in 6-well tissue culture dishes under standard tissue culture conditions. Test cells were pretreated for 18–24 hr with doxycycline, CMTs (1–50 μ g), or vehicle (DMSO 10 μ g/ml). IL-1 β was added to culture media at physiological relevant concentrations (10–20 pM), and the cells continued to incubate for an additional 18 hr. Time course experiments as well as dose response experiments with CMTs were conducted in the same manner. Mouse IL-6 secretion was measured by a capture ELISA kit (Quantikine, R&D Systems) following the manufacturer's protocol. Quantities of IL-6 were expressed in pg/ml of cultured supernatant.

^fAddress for correspondence: Keith L. Kirkwood, D.D.S. Ph.D., State University of New York at Buffalo, Department of Periodontics, 215 Squire Hall, 3435 Main Street, Buffalo, New York 14214-3000. Phone, 716/829-3845; fax, 716/837-7623; e-mail, klkirk@acsu.buffalo.edu

Northern Blot Analysis of Osteoblast Gene Expression

For detection of mRNA from control and CMT-treated MC3T3-E1 osteoblastic cells, we employed Northern blot using methods as described previously.³ Rat IL-6 cDNA clone was obtained as a gift from Dr. J. Goldie, McMaster University, Hamilton, Ontario. Dr. R. Franceschi, University of Michigan, kindly provided the mouse type 1(α 1) procollagen cDNA probe. After hybridization with the appropriate probes, membranes were washed at high stringency, and the hybridized probe was quantitated using Bio-Rad's phosphorimager system and Molecular Analyst software version 1.5.

RESULTS AND DISCUSSION

Chemically Modified Tetracyclines Inhibit IL-6 Secretion in MC3T3-E1 Cells

To determine if IL-6 secretion can be regulated by doxycycline or the other CMTs, IL-6 secretion from MC3T3-E1 cells was measured using a capture ELISA kit specific for mouse IL-6. Results shown in FIGURE 1A indicate that CMT-8 (10 μ g/ml) can inhibit IL-6 secretion from MC3T3-E1 cells when stimulated by IL-1 β (10 ng/ml). CMT-8 decreased IL-6 secretion by ~49% compared to cells pre-

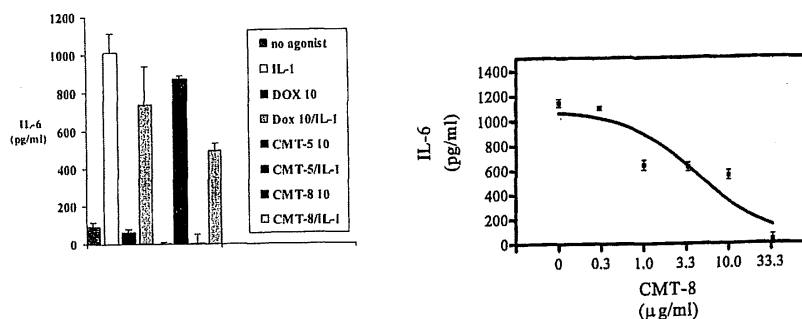


FIGURE 1. (Left) The effects of chemically modified tetracyclines on IL-1 β -induced IL-6 secretion in osteoblasts. MC3T3-E1 cells were pretreated for 18 hours with doxycycline or CMTs at 10 μ g/ml. Fresh media was added with the pretreatment conditions \pm IL-1 β (12.5 ng/ml). Cells continued to incubate for an additional 18 hours, and cells supernatants were harvested. IL-6 secretion was measured using an IL-6 Quantikine Kit (R&D Systems). Quantitation of IL-6 was made using linear regression with a standard curve. Each measurement was made in duplicate, and the mean of two different experiments was used for analysis with standard deviations. The results are considered statistically significant ($p = 0.005$). (Right) CMT-8 dose-response in IL-1 β treated MC3T3-E1 osteoblasts. MC3T3-E1 cells were pretreated with CMT-8 at the following concentrations: 0, 0.3, 1.0, 3.3, 10.0, and 30.0 μ g/ml. Cells were subsequently treated with IL-1 β (12.5 ng/ml) after a change in media and addition of pretreatment conditions. IL-6 measurements were made as described. Means of two separate experiments performed in duplicates \pm standard deviations of the mean. The IC_{50} was calculated to be 4.4 μ g/ml.

treated with vehicle. CMT-5 decreased IL-6 secretion by ~13%. Doxycycline decreased IL-1 β -induced IL-6 secretion by ~33%. The results shown are the means of two separate experiments performed in duplicate. Standard deviations are shown; these results are considered statistically significant ($p = 0.005$). Inhibition of IL-1 β -stimulated IL-6 secretion occurred in a dose-dependent manner with pharmacologically relevant concentrations of this drug (1–10 $\mu\text{g/ml}$) reducing IL-6 by about 50% as shown in FIGURE 1A. Pretreatment with CMT-8 was reduced to 8 hours in these experiments. FIGURE 1B illustrates the dose-response effect with decreasing amounts of IL-6 secretion when increasing the amount of CMT-8. The IC_{50} for CMT-8 inhibition of IL-1 β -induced IL-6 secretion was experimentally determined to be 4.4 $\mu\text{g/ml}$.

CMT-8 Decreases Steady State IL-6 mRNA Levels in MC3T3-E1 Cells

Northern blot analysis was used to determine IL-6 mRNA expression in IL-1 β -treated cells with or without pretreatment with doxycycline or CMTs. IL-6 mRNA species of 1.2–2.4 kb were detected in MC3T3-E1 cells (FIGURE 2). As shown previously in these cells, IL-1 β increases steady state mRNA levels. When cells are pretreated with doxycycline or CMTs or treated after IL-1 β stimulation (data not shown), CMT-8 decreases the IL-6 mRNA steady state levels in these cells. In contrast, neither doxycycline nor CMT-5 were able to decrease IL-6 mRNA steady state levels (FIG. 2). When levels of GAPDH were used to normalize the data, an approximate 50% reduction in IL-6 mRNA with CMT-8 treatment is seen compared to

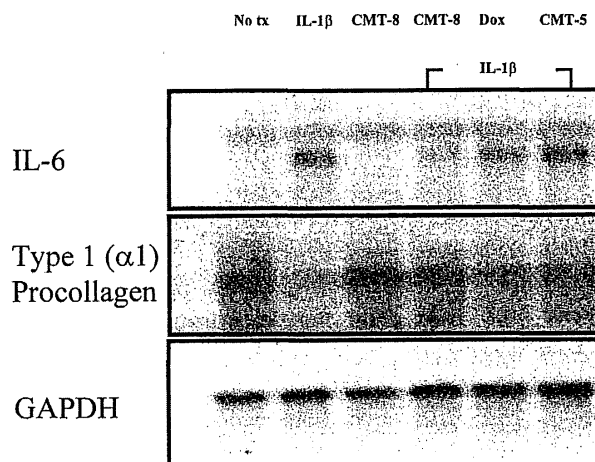


FIGURE 2. MC3T3-E1 cells were cultured in the presence or absence of CMTs of doxycycline and then exposed to IL-1 β (12.5 ng/ml) for 18 hours. Total RNA was purified, separated on agarose gels, blotted onto nylon membranes, and sequentially hybridized with [^{32}P]-labeled cDNA probes specific for rat IL-6, mouse type 1(α 1) procollagen, and rat GAPDH

IL-1 β treated cells ($n = 2$). Additionally, CMT-8 had no effect on constitutive type I procollagen $\alpha 1(I)$ gene expression in MC3T3-E1 cells. IL-1 β decreased type I procollagen $\alpha 1(I)$ gene expression by 54% in these cells. This effect was somewhat inhibited by CMT-8 (34%) and to a greater extent by doxycycline (76%).

The results of the present study suggest an additional mechanism of CMTs, inhibition of IL-6 gene expression, and secretion from osteoblasts. Our data indicate that IL-1 β -stimulated expression and secretion of IL-6 in MC3T3-E1 osteoblasts can be inhibited by CMT-8 in a dose-dependent manner, and the effect of CMT-8 is consistent with decrease of IL-6 gene expression. These results support the role for chemically modified tetracyclines to provide anabolic effects in bone. In this paper, CMT-8 and, to a lesser extent, doxycycline inhibited the potentially catabolic effects of IL-1 β , suggesting a novel molecular mechanism of these drugs with the treatment of metabolic bone diseases, such as osteoporosis.

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