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S. Gilbertson-Beadling · E.A. Powers · M. Stamp-Cole
P.S. Scott · T.L. Wallace · J. Copeland · G. Petzold
M. Mitchell · S. Ledbetter · R. Poorman
J.W. Wilks · C. Fisher

The tetracycline analogs minocycline and doxycycline inhibit angiogenesis in vitro by a non-metalloproteinase-dependent mechanism

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Abstract The tetracycline analogs minocycline and doxycycline are inhibitors of metalloproteinases (MMPs) and have been shown to inhibit angiogenesis in vivo. To further study the mechanism of action of these compounds we tested them in an in vitro model of angiogenesis: aortic sprouting in fibrin gels. Angiogenesis was quantitated in this system by a unique application of planar morphometry. Both compounds were found to potently inhibit angiogenesis in this model. To further characterize the activity of these compounds against MMPs, we determined the IC_{50}s of both compounds against representatives of three classes of metalloproteinases: fibroblast collagenase, stromelysin, and gelatinase A. Doxycycline was found to inhibit collagenase, gelatinase A and stromelysin with IC_{50}s of 452 μM, 56 μM and 32 μM, respectively. Minocycline was found to inhibit only stromelysin in the micromolar range with an IC_{50} of 290 μM. Since these results suggest that these compounds may not have been inhibiting in vitro angiogenesis by an MMP-dependent mechanism, we decided to test the effects of the potent MMP inhibitor BB-94. This compound failed to inhibit aortic sprouting in fibrin gels, thus strongly suggesting that both doxycycline and minocycline act by an MMP-independent mechanism. These results have implications for the mechanism of action of tetracycline analogs, particularly where they are being considered for the treatment of disorders of extracellular matrix degradation including periodontal disease, arthritis, and tumor angiogenesis.

S. Gilbertson-Breadling · E.A. Powers · M. Stamp-Cole · P.S. Scott
T.L. Wallace · J. Copeland · G. Petzold · S. Ledbetter · R. Poorman
J.W. Wilks · C. Fisher (✉)
Cancer and Infectious Diseases Research, Upjohn Laboratories, The Upjohn Company, 7252-267-412, Kalamazoo, MI 49001, USA

M. Mitchell
Medicinal Chemistry Research, Upjohn Laboratories, The Upjohn Company, 7252-267-412, Kalamazoo, MI 49001, USA

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Introduction

The formation of new blood vessels, or angiogenesis, is important in the pathogenesis of a number of disorders including cancer in which tumor growth is dependent upon neovascularization [9]. Angiogenesis is believed to occur by sprouting of new vessels from established ones [1], and to involve the proliferation and migration of vascular cells, including endothelial cells, in response to tumor angiogenic factors [18]. The production of matrix degrading enzymes is thought to mediate this process by facilitating penetration of the new vessels into the extracellular matrix (ECM). One class of enzymes implicated in this process is the matrix metalloproteinases (MMPs).

The MMPs are a family of enzymes that degrade various ECM components [17]. Interstitial collagenase, the most specific MMP, initiates type I collagen degradation via a single cleavage of the triple helical molecule [7, 19]. The gelatinases degrade gelatin (denatured collagen) and type IV collagen [27], while stromelysin is the most broadly acting, degrading laminin, proteoglycans, gelatin, and a variety of other ECM components [17, 32]. MMP production is induced in activated, migratory endothelial cells [22], and inhibition of MMP production has been shown to block angiogenesis in vitro [8]. Such observations suggest that MMPs might be good targets for antiangiogenic therapy, and recent reports have provided support for this concept [6, 15].

Doxycycline and minocycline, two analogues of tetracycline, inhibit tumor-induced angiogenesis in the rabbit cornea [28]. In addition, these analogues have been reported to specifically suppress endothelial cell growth [13]. Because they inhibit MMP activity, primarily in assays involving crude cell preparations or

clinical samples [10, 14, 31, 33] and in assays utilizing purified enzyme [2, 26, 27], an implicit assumption exists that minocycline and doxycycline inhibit angiogenesis via an MMP-dependent mechanism (see, for example, references 26, 30, 31). Tetracycline analogues reportedly are potent inhibitors of neutrophil collagenase [28, 29] and gelatinase [12], but are poor inhibitors of fibroblast collagenase [28, 29]. However, a systematic assessment of minocycline and doxycycline activity against a variety of MMPs is lacking, as is a critical evaluation of their activity against MMPs in in vivo or in vitro models of ECM remodelling.

To examine the effects of potential inhibitors of angiogenesis in vitro, we established a unique system to quantitate aortic sprouting in fibrin gels. We found both minocycline and doxycycline to be potent inhibitors in this system. We then examined the role of MMPs in this process by determining the IC_{50}s of both compounds against purified interstitial collagenase, stromelysin, and gelatinase A. Doxycycline had modest activity against gelatinase A and stromelysin but was a poor inhibitor of fibroblast collagenase. Minocycline was largely ineffective against all three enzymes. Finally, we determined that an MMP inhibitor effective at nanomolar concentrations against angiogenesis in vitro in collagen gels [8] did not suppress angiogenesis in the aortic sprouting model. Our results suggest that doxycycline and minocycline are potent inhibitors of in vitro angiogenesis acting by a non-MMP-dependent process.

Materials and methods

Tissue preparation and culture

Male Fischer rats (Charles River, Kingston, N.Y.) were sacrificed at 3–4 weeks of age by chloroform overdose. The descending aortae were dissected out and rinsed in Hanks' balanced salt solution (HBSS; Gibco BRL). The tunica adventitia, attached fat, and any clotted blood were removed from the aortae before sectioning them into 1–2-mm rings. The rings were washed in minimal essential medium (MEM; Gibco BRL) containing 20% fetal bovine serum (FBS) and 50 μg/ml gentamicin then embedded into preformed fibrin clots. In general, three to six aortic rings were included in each treatment group.

Fibrin clot formation:

Fibrin clots were formed in 24-well (16 mm) culture dishes (Corning, Corning, N.Y.) following the addition of 1 unit thrombin to 1 ml culture medium (MEM with 20% FBS and gentamicin, as above) containing 3 mg/ml human fibrinogen (Kabi Vitrum, Stockholm, Sweden). The clots gelled at room temperature shortly after the addition of the thrombin, and were then incubated in air containing 5% CO_2 at 37°C until the aortic ring was embedded. After embedding the aortic ring, 1 ml culture medium per well was added. The culture medium was renewed every 2–3 days. Minocycline and doxycycline were added at the indicated concentrations to both the fibrinogen prior to clotting, and to the medium during the entire period of culture.

Quantitation of sprouting:

The rings were photographed on days 2–5 using a Zeiss inverted microscope on Ilford 50 ASA black-and-white film. A 3.5×5 inch micrograph was used to quantitate sprouting using a Microcomp Image Three analysis system (Microcomp Southern Micro Instruments, Atlanta, Ga.). The system was calibrated before each analysis using a micrograph of a stage micrometer taken at the same magnification as the aortic rings. Individual micrographs of the aortic rings at the various time-points were overlaid with a circular grid having eight lines radiating from the center, and then placed on a digitizing tablet. The distance from the outer edge of the aortic ring to the farthest point of growth was measured at each of the eight points using a mouse, and the average distance of outgrowth of sprouts calculated. A statistical program (kindly prepared by Norm Young, Upjohn Laboratories) was used to determine the mean, standard deviation and standard error of the mean (SEM) for all aortic ring specimens in all treatment groups.

Metalloproteinase assays

Collagenase assay

Human interstitial collagenase (MMP-1) cDNA cloned into the EcoRI site of pUC19 (kindly provided by C. Brinckerhoff, Dartmouth), encoding all but the 48 aminoterminal amino acids of full-length procollagenase, was put in frame relative to the lac promoter. The IPTG-induced collagenase was then purified from *E. coli* inclusion bodies by anion exchange chromatography, following solubilization in 6 *M* guanidine-HCl and dialysis into 8 *M* urea. The enzyme was activated with stromelysin prior to assay. This assay was modified from the procedure of Cawston and Barrett [4] and has been reported elsewhere in more detail [8]. Rat tail ^3H collagen was diluted into one part $10 \times$ Tris buffer (500 m*M*, pH 7.5) and eight parts cold collagen (~ 1.2 mg/ml) and the pH adjusted to 7.5. Aliquots (50 μl) were gelled overnight at 37°C in microfuge tubes. Activated collagenase (0.5 μg) was added and the tubes incubated for 24 h at 37°C, centrifuged at 13,000 *g* at 22°C for 10 min, and 50 μl of the supernatants was collected and counted in 10 ml Ready Safe Liquid Scintillation Cocktail (Beckman, Fullerton, Calif.) for 3 min in a Beckman liquid scintillation counter.

Gelatinase A assay

Human gelatinase A (MMP-2) cDNA (kindly provided by W. Stetler-Stevenson, NIH) was subcloned into the baculovirus vector PVL1393. A high-level-expressing clone was selected from transfected Sf9 cells. Conditioned medium containing the expressed 72-kDa gelatinase A protein was concentrated on a S1Y10 Amicon filter and purified by chromatography on a gelatin-sepharose column and a cation exchange column. Then 25 μl of heat denatured substrate (20,000 cpm/well ^3H type I collagen and 2 ng/μl cold type I collagen) was incubated with 10 ng gelatinase A in 25 μl buffer (50 m*M* Tris-HCl, pH 7.4, 150 m*M* NaCl, 20 m*M* $CaCl_2$, and 0.02% NaN_3). Test compounds were added in 1-μl aliquots of a $50 \times$ stock solution made in dimethyl sulfoxide (DMSO). The contents of the plate were mixed, incubated for 4 h at 37°C, and precipitated with trichloroacetic acid (TCA) for 15 min at − 20°C. The plate was centri-

fuged at 3000 *g* for 15 min, and 100 µl of the supernatant was mixed with 10 ml Ready Safe Liquid Scintillation Cocktail, and counted in a Beckman liquid scintillation counter.

Stromelysin assay

Recombinant human stromelysin-1 (MMP-3) lacking the hemopexin domain was expressed in *E. coli* strain BL21(DE3)(pLysE) in plasmid pET3c. Bacteria expressing stromelysin were kindly provided by P. Kaytes and G. Vogeli (Upjohn Laboratories). The enzyme was purified according to previously published procedures [16]. Then 25 µl ^{14}C-β-casein (25,000 cpm/well) was pipetted into round bottomed polystyrene 96-well plates, and 25 µl chymotrypsin-activated stromelysin (0.4 µg/ml) added. Plates were incubated overnight (18–20 h) in a humidified incubator in room air at 37°C. The samples were TCA precipitated using bovine serum albumin as a carrier. Following centrifugation, supernatants from each well were transferred to Packard 96-well picoplates containing 150 µl Packard Microscint-40 scintillant, the plates mixed by shaking, and the counts released from the ^{14}C-β-casein by stromelysin determined.

MMP inhibitor, and tetracycline analogs

BB-94, a peptide-based inhibitor of MMPs [5], was synthesized according to methods previously described [3]. The compound shows strong inhibitory activity against all MMPs (Table 1) [3, 5, 8]. Doxycycline and minocycline were obtained from Sigma Chemical Company, St. Louis, Mo.

Results

Sprouting of aortic rings and inhibition with tetracycline analogs

Aortic rings in culture formed microvascular-like sprouts over the course of several days. The sprouts were composed of overlapping cells that extended radially from the aortic ring (Fig. 1). This process was found to progress over time, but by day 7 of culture the fibrin was highly degraded. The degree of sprouting varied between experiments in both the total number and the length of sprouts, but within a given experiment these factors tended to remain consistent. Quantitation of sprouting (Figs. 2–4) indicated that growth was linear over 4 days.

Inhibition of sprouting with tetracycline analogs

Culturing the aortic rings in the presence of 100 µ*M* doxycycline (Fig. 2) or minocycline (Fig. 3) resulted in the complete inhibition of aortic sprouting, while 50 µ*M* of the drugs resulted in partial inhibition (Figs. 2 and 3). Lower concentrations of doxycycline (Fig. 2) and minocycline (Fig. 3) had no measurable effect on sprouting relative to controls.

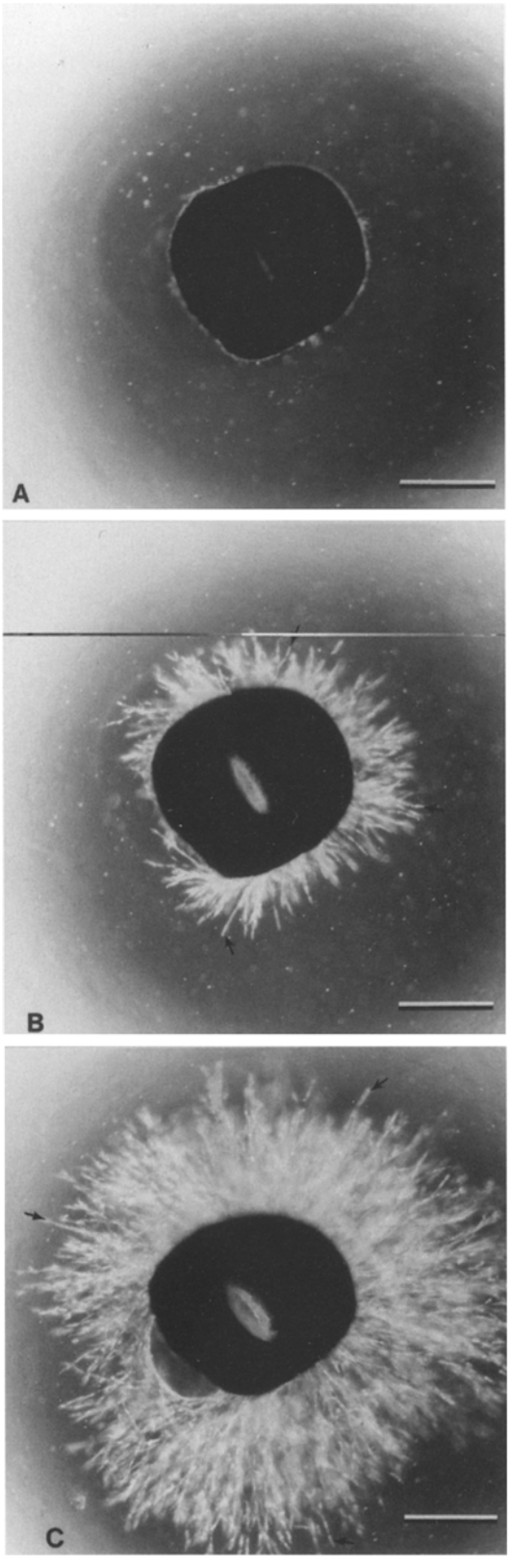

Fig. 1A–C Photomicrographs of sprout formation from aortic rings on day 2 (**A**), day 3 (**B**), and day 4 (**C**) of culture. *Arrows* in **B** and **C** indicate individual sprouts (*bar* = 500 µm)

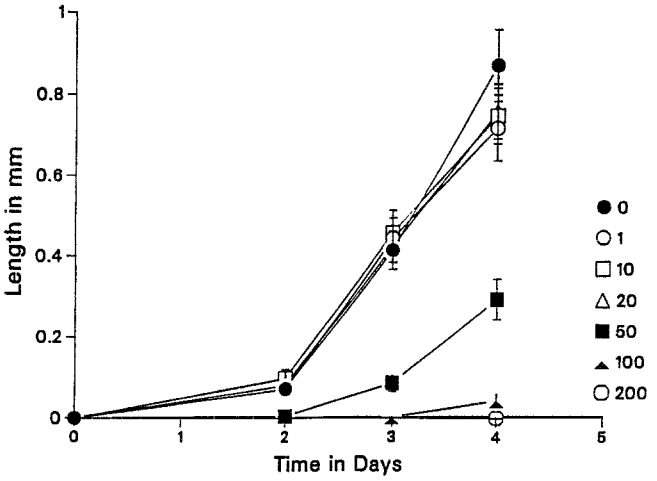

Fig. 2 Inhibition of aortic sprouting with doxycycline. Doxycycline at concentrations of 50 μ*M* and higher result in the inhibition of sprouting

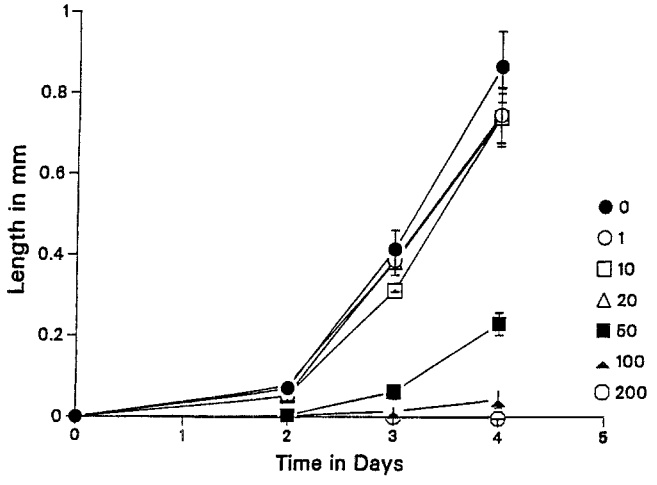

Fig. 3 Inhibition of aortic sprouting with minocycline. Minocycline at concentrations of 50 μ*M* or higher can be seen to inhibit aortic sprouting

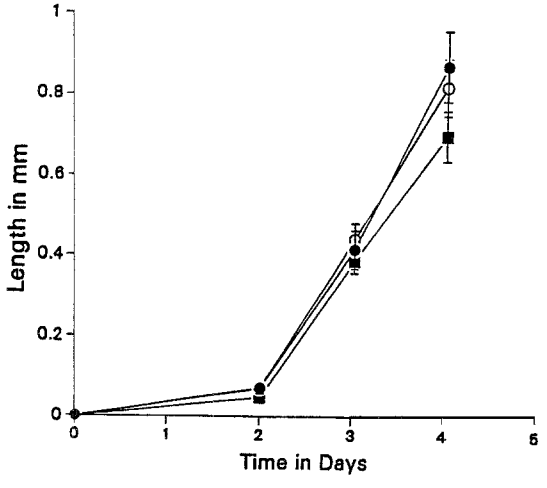

Fig. 4 Photomicrographs of aortic sprouting in presence of 10 μM (A), 50 μM (B), and 200 μM (C) minocycline. Bar = 500 μM (0.5 mm)

Table 1 IC_{50}s of doxycycline, minocycline and BB-94 against matrix metalloproteinases

	Doxycycline	Minocycline	BB-94
Collagenase	452 ± 26 μM	> 10 mM	21 ± 4 nM
Gelatinase A	56 ± 7 μM	1.3 ± 0.4 mM	0.8 ± 0.01 nM
Stromelysin	32 ± 3 μM	290 ± 23 μM	1.2 ± 0.06 nM

MMP IC_{50}s of tetracycline and doxycycline

Inhibition of aortic sprouting within a fibrin ECM by minocycline and doxycycline suggests that these compounds may be acting on MMPs. To examine this possibility further, we determined the IC_{50} for both compounds against interstitial collagenase, gelatinase A, and stromelysin (Table 1). Doxycycline exhibited an IC_{50} of 452 μ*M* against collagenase, while the IC_{50} for minocycline was 10 m*M*. The gelatinase A IC_{50} was 56 μ*M* for doxycycline but 1.3 m*M* for minocycline. Stromelysin was inhibited by doxycycline and minocycline with IC_{50}s of 32 μ*M* and 290 μ*M*, respectively. Thus, aortic sprouting was inhibited at concentrations of both minocycline and doxycycline below their respective IC_{50}s against the three MMPs tested.

BB-94 does not inhibit sprouting

If minocycline or doxycycline were acting through an MMP-dependent process then other MMP inhibitors might be expected to show similar effects. For this reason BB-94, an inhibitor of MMPs with nanomolar to subnanomolar potency (Table 1) [3, 5, 8] was tested for its ability to suppress sprouting. No significant inhibition of aortic sprouting was noted at a concentration 1 μ*M* (Fig. 5).

Discussion

Tetracycline analogs are efficacious against a number of disorders in which ECM destruction or remodelling plays an important etiological role such as periodontal disease [10], rheumatoid arthritis [12] and tumor-induced angiogenesis [30]. Initial reports indicated that tetracycline analogues inhibit collagenolytic activity [10, 11], and subsequent reports have described the inhibition of collagenolytic activity of cell culture supernatants, clinical samples, and purified enzyme [2, 10–12, 14, 28, 29]. MMPs directly inhibited by tetracycline analogues include neutrophil collagenase and gelatinase [12, 28, 29].

That doxycycline is reportedly more potent against neutrophil collagenase than fibroblast collagenase

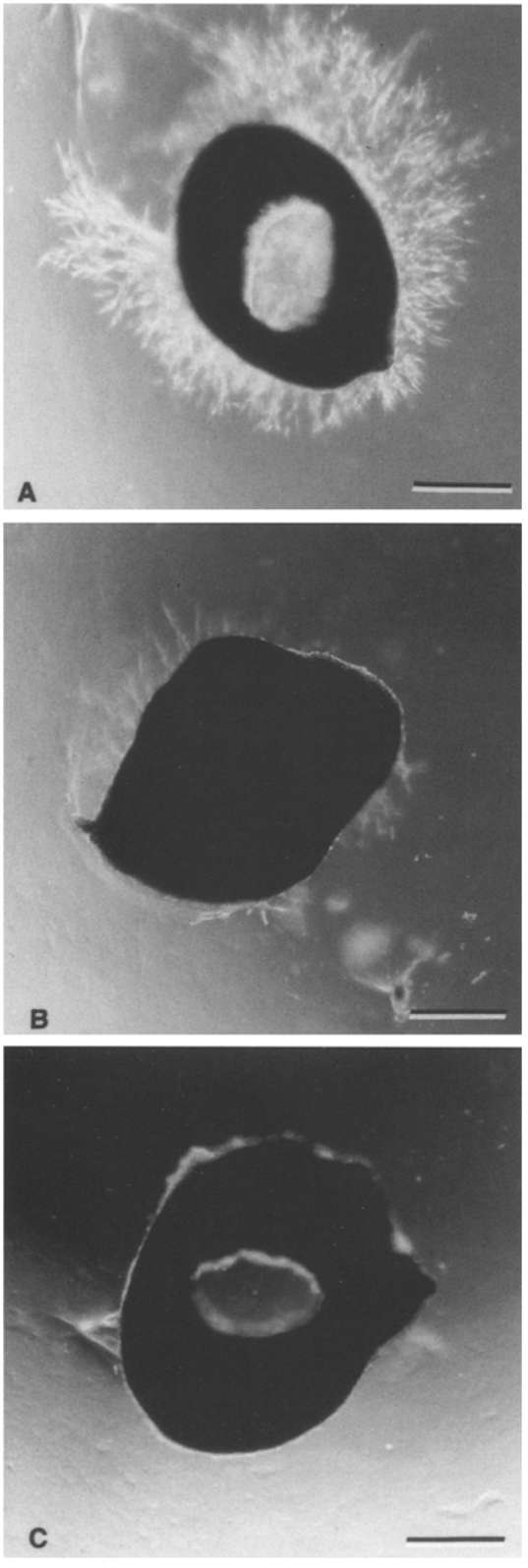

Fig. 5A–C Photomicrographs of aortic sprouting in the presence of 10 μ*M* (**A**), 50 μ*M* (**B**), and 200 μ*M* (**C**) minocycline (*bar* = 500 μm)

suggests that variability in relative potency of the tetracycline analogs for various MMPs might exist. The results presented here offer the first systematic evaluation of tetracycline analog inhibition against members of multiple classes of MMPs. Our results suggest that doxycycline has significant activity against two MMPs, stromelysin and gelatinase A, but not against interstitial collagenase (Table 1). The IC_{50} of 452 μ*M* determined for doxycycline against fibroblast collagenase is significantly greater than that of 280 μ*M* previously reported [28, 29]. The difference may be explained by experimental differences between the two assays. While both assays utilized purified enzymes, our assay utilized native, insoluble collagen at physiological temperature while Suomalainen et al. [28, 29] utilized a soluble substrate at 22°C. Minocycline was less potent than doxycycline, inhibiting stromelysin and gelatinase A with IC_{50}s of 290 μ*M* and 1.3 m*M*, respectively, while its IC_{50} against interstitial collagenase was above our highest concentration tested (1 m*M*). These results suggest that some biological activities of tetracycline analogs previously ascribed to MMP inhibition may be due to alternative activities of the drugs. We examined the activity of minocycline and doxycycline against angiogenesis in vitro in more detail.

The sprouting of aortic rings in fibrin gels accurately reproduces important aspects of angiogenesis in vivo [20, 24, 25]. We developed a method to accurately quantitate aortic sprouting in three-dimentional gels using planar morphometry. Using this method both minocycline and doxycycline were found to suppress sprouting at a concentration of 50 μ*M* or higher. This potency is in agreement with a previous report demonstrating the selective inhibition of endothelial cell growth by tetracycline analogs [13]. Our results are also consistent with those of Tamargo et al. [30] who showed that minocycline inhibited angiogenesis in vivo in a rabbit cornea model. That both minocycline and doxycycline inhibit aortic sprouting in vitro suggests that they are working by a mechanism of action similar to that seen in the above studies. However the lack of potency of minocycline against all tested MMPs suggests that both compounds may be acting by a non-MMP-dependent mechanism to suppress aortic sprouting. This possibility was examined by testing the effects of BB-94, a potent MMP inhibitor [3, 5, 8], on aortic sprouting in fibrin gels.

Previous studies have demonstrated that BB-94 completely inhibits collagenolytic activity, invasion, and formation of microvascular-like structures of endothelial cells grown on collagen gels [8]. However, BB-94 did not inhibit aortic sprouting in the present study, suggesting that this process was not MMP dependent. These results are consistent with those of Pepper et al. [25] who demonstrated that serine proteases of the plasminogen activator/plasmin cascade are the predominant proteolytic system mediating angiogenesis

in vitro in fibrin gels. For this reason we conclude that both minocycline and doxycycline inhibit angiogenesis in vitro by a non-MMP-dependent mechanism.

Our data do not preclude the action of tetracycline analogues against MMPs during angiogenesis in vivo. The potent inhibitory activity of these compounds against neutrophil collagenase might be expected to play a more important role against inflammation-mediated angiogenesis. However, our data suggest that previous studies examining the antiangiogenic effects of tetracycline analogs based on an assumed MMP-dependent mechanism of action (see references 26, 30 and 31) should be re-evaluated.

The mechanism of action of these agents in the present study is unclear. One possibility is that minocycline and doxycycline act by chelation of divalent cations [23]. This activity might be expected to attenuate cell signalling pathways by buffering Ca^{2+}. The inhibition of ouabain-induced cyclic AMP accumulation by 50 μM minocycline [21] is consistent with this activity. Further studies on the mechanism of action of the tetracycline analogs are clearly needed before we will begin to understand their antiangiogenic activity.

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