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Bioorganic & Medicinal Chemistry 15 (2007) 2368–2374
Bioorganic & Medicinal Chemistry

Synthesis and in vitro evaluation of targeted tetracycline derivatives: Effects on inhibition of matrix metalloproteinases

Aurélien Vidal,[a,b,c,*] Massimo Sabatini,[d] Gaëlle Rolland-Valognes,[d] Pierre Renard,[d] Jean-Claude Madelmont[a,b,c] and Emmanuelle Mounetou[a,b,c]

[a]*INSERM 484, Clermont-Ferrand F-63500, France*
[b]*Univ d'Auvergne, Clermont-Ferrand F-63500, France*
[c]*Centre Jean Perrin, Clermont-Ferrand F-63500, France*
[d]*Institut de Recherches Servier, Suresnes F-92150, France*

Received 13 July 2006; revised 22 December 2006; accepted 17 January 2007
Available online 19 January 2007

Abstract—Among other non-antibiotic properties, tetracyclines inhibit matrix metalloproteinases and are currently under study for the treatment of osteoarthritis. Quaternary ammonium conjugates of tetracyclines were synthesized by direct alkylation of the amine function at the 4-position with methyl iodide. When tested in vitro, they inhibited cytokine-induced MMP expression to a lesser extent than parent tetracyclines. This was compensated by an improved inhibition of MMP catalytic activity. Since inhibition of collagen degradation was maintained these derivatives could be potent drug candidates for cartilage-targeted chondroprotective treatment.

1. Introduction

Matrix metalloproteinases (MMPs) play a major role in cartilage metabolism as degradative enzymes of the extracellular matrix, which is mainly formed by proteoglycans and collagen. Both synthesis as pro-enzymes and activation to the mature form are upregulated by pro-inflammatory cytokines, including interleukin-1β (IL-1β).[1] Activated MMPs can be inhibited by endogenous tissue inhibitors of metalloproteinases (TIMPs), which non-covalently bind MMPs with high affinity.[2,3] Due to the central role of MMPs in a wide number of pathological processes, MMP inhibition is regarded as a promising therapeutic approach, particularly for the treatment of osteoarthritis.[4] Aside from their well-known anti-microbial activity, tetracyclines inhibit MMPs through actions at three levels: mRNA expression, pro-enzyme activation, and catalytic activity of the mature enzyme.[5–10]

The anti-MMP activity of tetracyclines slows down the degradation of articular cartilage, which is generally increased in arthritic pathologies. A first clinical trial of doxycycline (DC) on patients with osteoarthritis showed positive effects on both the symptoms and clinical signs of cartilage loss.[11,12] However, long-term administration of tetracyclines, which is essential in treating slow-developing degenerative pathologies such as osteoarthritis, can lead to severe adverse effects (kidney and liver damage), partly due to the antibiotic properties of those molecules.[13,14]

Our strategy was based on drug targeting toward cartilage via the introduction of a quaternary ammonium function (QA), which displays a high affinity for the anionic sites (carboxylate and ester sulfate groups) of proteoglycans.[15] This concept has already led to the development of cartilage-selective radiopharmaceuticals[16,17] and drugs.[18–22] In the present study, it was applied to tetracycline (TC) and DC by quaternization of the C-4 dimethylamino group. This approach was supported by structure–activity studies demonstrating that the antibiotic properties of tetracyclines are dependent on this C-4 dimethylamino group, whereas the inhibition of MMP catalytic activity is more due to the oxygens of the lower part of the molecule.[23] Our

Keywords: Tetracyclines; Quaternary ammonium; Osteoarthritis; Metalloproteinases.
* Corresponding author. Tel.: +33 4 73 15 08 18; fax: +33 4 73 15 08 01; e-mail: vidal@inserm484.u-clermont1.fr

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doi:10.1016/j.bmc.2007.01.026

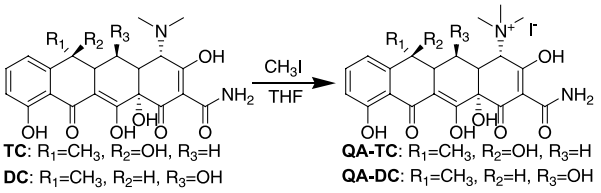
objective was to develop more selective anti-MMP drugs that would concentrate strongly in cartilaginous tissues and thus allow a significant decrease of the effective dose and, as a consequence, of general side effects. The results of this structural modification on the anti-MMP properties of TC and DC were analyzed in vitro with respect to mRNA expression of MMP-3 and MMP-13, the production of MMP-13 in chondrosarcoma cells, the catalytic enzymatic activity of MMPs on a synthetic substrate and on articular cartilage explants, and finally, collagen degradation on articular cartilage explants.

2. Results and discussion

2.1. Chemistry

The quaternized conjugates of TC (QA-TC) and DC (QA-DC) were prepared by direct methylation of the C-4 dimethylamino group of TC and DC free bases according to Scheme 1. Physical data and spectral characterizations of QA-TC and QA-DC are presented in the Section 4.

NMR proved to be particularly useful in clarifying the chemical structures of various forms of tetracyclines (hydrochloride, free base).[24,25] 1D (^{1}H, ^{13}C) and 2D (COSY, HMQC) experiments performed at 500 MHz enabled complete proton and carbon assignment for each QA derivative in DMSO-d_6, in accordance with the data reported by Asleson and Casy for TC and DC.[24,25] In the ^{1}H NMR spectra, two intense signals at 1.50 (s) and 3.40 (s) ppm for QA-TC and at 1.42 (d) and 3.35 (s) ppm for QA-DC were assigned to the C-6 methyl and C-4 trimethylammonium groups, respectively. The presence of a hydroxyl group at C-5 in QA-DC did not influence the chemical shift of the C-4 proton, as previously noted for DC.[25] Moreover, the two H-5 methylene protons were non-equivalent in QA-TC. The H-5 methylene signal at 1.72 ppm that displayed a broad quadruplet pattern was assigned to the pseudo-axial β-proton, whereas the broad doublet at 2.25 ppm was assigned to the pseudo equatorial α-proton, in accordance with Casy.[25] Broad resonances at 5.02, 7.71, 9.43, 9.51, 11.74, and 15.24 ppm for QA-TC and at 5.69, 7.67, 9.09, 9.22, 11.45, and 15.26 ppm for QA-DC were assigned to amino and hydroxy groups, since they disappear in samples that have been deuterium exchanged. Moreover, the two NH_2 protons were non-equivalent in QA-TC and QA-DC. Finally, the signals of the aromatic protons were observed, as expected, as a triplet and two doublets at 7.55, 6.91, 7.12 and 7.53, 6.85, 6.89 ppm for QA-TC and QA-DC, respectively. In ^{13}C NMR spectra, the three trimethyl carbons of the ammonium function were observed as one signal at 54.75 ppm for QA-TC and 55.56 ppm for QA-DC. A HMQC sequence allowed to clearly distinguish the C-4 signal from C-6 and C-12a signals for QA-TC. The signal at 68.79 ppm was assigned to the C-6 and the signal at 73.09 ppm to the C-12a based on comparison with the TC spectrum.[24] For QA-DC, the same HMQC sequence allowed to distinguish the C-4 signal (74.92 ppm) from C-5 (69.10 ppm) and the C-4a signal (46.56 ppm) from C-5a (44.33 ppm).



Scheme 1. Synthesis of QA derivatives.

2.2. Biological activity

2.2.1. mRNA expression and production of MMPs. TC, DC, and QA derivatives were first tested for their ability to inhibit IL-1β-induced mRNA expression of MMP-3 and MMP-13 in human SW-1353 chondrosarcoma cells (Fig. 1). RT-PCR analysis showed that IL-1β strongly stimulated the mRNA expression of MMP-3 and -13 compared with the control. This effect of the cytokine was totally blocked by 10^{-4} M TC or DC. QA-TC and QA-DC were more effective in inhibiting the mRNA expression of MMP-13 than MMP-3, but this selective activity against MMP-13 remained weaker than that of the parent tetracyclines at the same concentration. Moreover, QA-DC was more potent than QA-TC. On one hand, this loss of activity could be explained by a reduced cellular uptake due to the positive charge introduced by the quaternary ammonium function. On the other hand, a region of the tetracycline involved in the inhibition of MMP expression may have been modified by the quaternization. However, this second hypothesis could not be confirmed, since no structure–activity data on the effects of tetracyclines on MMP expression at mRNA level have been reported in the literature. This study on MMP expression at the mRNA level was complemented by experiments on MMP-13 protein levels, which were measured by ELISA. The activation of the mRNA expression of MMP-13 by IL-1β in the SW-1353 cells was paralleled by a large increase in enzyme release in the culture media (from 0.24 ± 0.09 to 70.11 ± 4.73 ng/mL; $P < 0.001$). At the highest tested

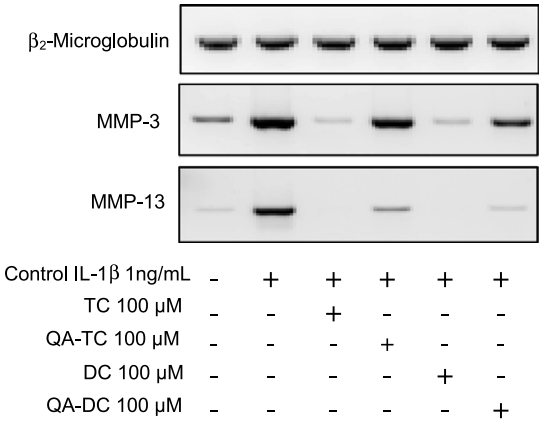


Figure 1. Effects of DC, TC, and their quaternized conjugates (QA-DC, QA-TC) on the IL-1β-induced mRNA expression of MMP-3 and MMP-13 in SW1353 human chondrosarcoma cells.

concentration of 50 μM, TC and DC but not their QA derivatives significantly inhibited IL-1β-induced MMP-13 production in SW-1353 cells (data not shown).

2.2.2. MMP catalytic activity. We evaluated the inhibitory effect of the tetracyclines on the catalytic activity of the following MMPs: gelatinase A/MMP-2, stromelysin-1/MMP-3, collagenase-2/MMP-8, and collagenase-3/MMP-13 (Table 1). The IC_{50} values were in the order of 10^{-4} M for TC and QA-TC, and 10^{-5} M for DC and QA-DC. QA-TC and QA-DC increased MMP-2, -8, and -13 inhibition by 50–64% and 182–716%, respectively. Conversely, the quaternized conjugates were less efficient inhibitors of MMP-3 than their parent tetracyclines. These diverging results on MMP activity and expression show that different interactions take place between tetracyclines and (a) MMP proteins and (b) targets, still unknown, controlling MMP expression. Also, they suggest that introduction of QA in C-4 can modulate the anti-MMP activity known to be exerted by the lower part of tetracycline molecules.[23] We used as a positive control the reference compound AG3340 (prinomastat), a potent broad-spectrum inhibitor of MMPs, with K_i values between 3×10^{-11} and 3×10^{-10} for MMP-2, -3, -9, -13, and -14.[25] In this study, AG3340 inhibited MMP-2, -3, -8, and -13 with IC_{50} values of 4×10^{-10}, 1×10^{-9}, 2×10^{-10} and 9×10^{-10} M, respectively.

Table 1. Effects of DC, TC, and their quaternized conjugates (QA-DC, QA-TC) on MMP-2, -3, -8, and -13 activity

	IC_{50}[a] (μM)			
	MMP-2	MMP-3	MMP-8	MMP-13
TC	220	140	180	180
QA-TC	140	150	110	120
Activity enhancement[b] (%)	+57	−7	+64	+50
DC	24	25	31	40
QA-DC	7.7	81	11	4.9
Activity enhancement[b] (%)	+212	−69	+182	+716

[a] Dose required to inhibit MMP activity by 50%.
[b] See Section 4.

2.2.3. Proteoglycan degradation. The anti-degradative effects of tetracyclines were examined on cultured fragments of rabbit articular cartilage. In order to activate the pro-MMPs induced by IL-1β, tissue fragments already stimulated by the cytokine were further incubated in the presence of the MMP activator APMA. Two protocols were used: one to examine the effects of tetracyclines on degradation depending on de novo MMP production, the other to study the effects on degradation depending on already active MMPs. In the first case (ability of tetracyclines and their QA conjugates to inhibit the production of IL-1β-induced MMPs) the fragments were stimulated for 24 h by IL-1β in the absence or presence of tetracyclines, and then exposed to APMA for another 24 h (Fig. 2a). IL-1β combined

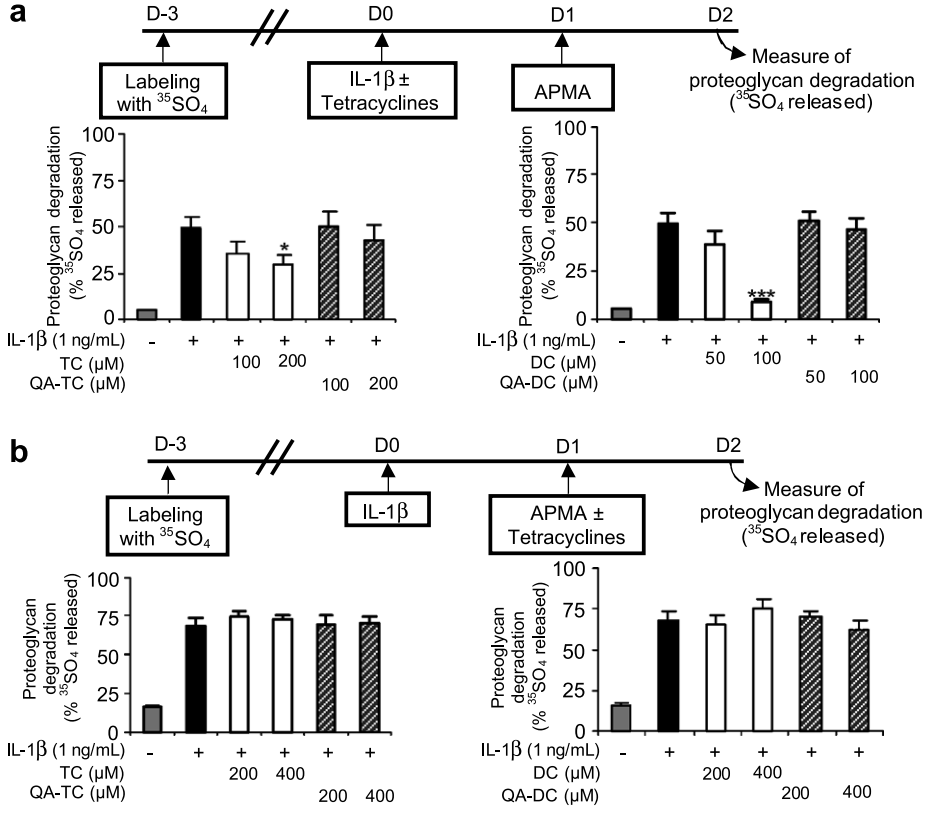


Figure 2. Effects of DC, TC, and their quaternized conjugates (QA-DC, QA-TC) on proteoglycan degradation induced by IL-1β and APMA, in fragments of rabbit articular cartilage. (a) Effects of the tetracyclines and their conjugates on IL-1β-induced MMP catabolic activity. (b) Effects of the same tetracyclines and their conjugates on the catabolic activity of MMPs previously induced by IL-1β. Degradation is expressed as percentage of radioactivity released from fragments previously labeled with $^{35}SO_4$. Data are expressed as means ± sem; $n = 8$/group. The asterisks indicate a significant difference between treated and IL-1β alone. $^{*}P < 0.05$; $^{***}P < 0.001$.

with APMA strongly stimulated proteoglycan degradation. A significant reduction of proteoglycan degradation induced by IL-1β plus APMA was observed for TC at 200 μM, with levels decreasing from 49% (control) to 29%, that is, a 45% ($P < 0.05$) inhibition of proteoglycan degradation. The effect was even stronger with 100 μM DC, with only 8% of proteoglycan degradation compared to 49% for the control, that is, a 93% inhibition of proteoglycan degradation. However, no inhibition was observed in the presence of the two QA derivatives used at the same concentrations as their parent compounds. In order to investigate whether tetracyclines could inhibit the catalytic activity of the MMPs already produced, cartilage fragments were stimulated by IL-1β for 24 h and then exposed to APMA in the absence or presence of tetracyclines (Fig. 2b). When tissue fragments were exposed to IL-1β followed by APMA, proteoglycan degradation was significantly stimulated. Nevertheless, no significant inhibition of proteoglycan degradation by MMPs was observed at concentrations reaching 400 μM, whatever the nature of the tetracycline. Conversely, in a separate experiment, the reference MMP-inhibitor AG3340 significantly inhibited proteoglycan degradation from a value of 87 ± 1% for IL-1β plus APMA, to 14 ± 1% at the concentration of 0.1 μM ($P < 0.001$).

This study on the inhibition of proteoglycan degradation depending on previously produced MMPs showed that none of the tetracyclines, either parent molecules or derivatives, could significantly decrease the catabolic action of the pre-produced enzymes, at concentrations ranging up to 400 μM. The very intense proteoglycan degradation observed with this protocol could nevertheless be inhibited, but only with a very potent MMP inhibitor such as AG3340, with IC_{50} values in the 10^{-10}–10^{-9} M range, compared with IC_{50} values for tetracyclines between 10^{-6} and 10^{-4} M. This means that, at least for proteoglycan degradation, the action of tetracyclines at concentrations in the range of 100 μM depends more on the blockage of MMP production than on the inhibition of the catalytic activity of pre-existing enzymes.

2.2.4. Collagen degradation. This experiment evaluated the effect of tetracyclines on both the production and catabolic activity of MMPs. In this assay, collagen degradation was dependent on both the induction of pro-MMPs by IL-1β and their activation by plasmin. When cartilage fragments were cultured in the presence of IL-1β and plasmin, collagen degradation was stimulated. DC and QA-DC significantly inhibited IL-1β- and plasmin-induced collagen degradation by 89% and 66%, respectively, at a concentration of 100 μM (Fig. 3). In addition, AG3340 significantly inhibited collagen degradation from a value of 54 ± 1% for IL-1β plus plasmin, to 15 ± 1% at the concentration of 10^{-7} M ($P < 0.001$) (data not shown).

In contrast to the results on proteoglycan degradation, QA-DC was effective at inhibiting collagen degradation, even though it was slightly less active than DC. Collagen degradation in cartilage appears mainly to depend on

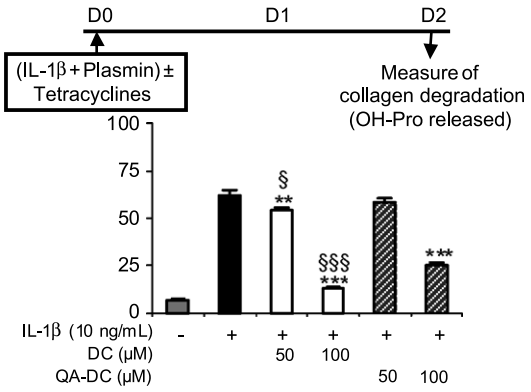


Figure 3. Effects of DC, TC, and their quaternized conjugates (QA-DC, QA-TC) on IL-1β-induced collagen degradation, in the presence of plasmin, in fragments of rabbit articular cartilage. The degradation is expressed as percentage of collagen released. Data are expressed as means ± sem; $n = 8$/group. Statistical significance: $^{**}P < 0.01$; $^{***}P < 0.001$ between treated and IL-1β. $^{\S}P < 0.05$; $^{\S\S\S}P < 0.001$ between DC and QA-DC at the same concentration.

MMP-13/collagenase-3, particularly in situations of increased tissue catabolism. MMP-13 has been shown to degrade type II collagen, which is the main component in cartilage, more rapidly than MMP-1 and MMP-8, and to be upregulated in chondrocytes from patients with osteoarthritis.[26,27] Moreover, overexpression of MMP-13 alone could cause osteoarthritis-type lesions in transgenic mice.[28] In contrast with the two assays of proteoglycan degradation, where inhibition is due to decrease of MMP production in one case, and of MMP activity in the other, the assay of collagen degradation can detect the effect of an inhibitor acting on either or both of MMP activity and MMP production. Compared with DC, QA-DC partially lost the ability to inhibit MMP-13 expression, while improving the effect against the catalytic activity of MMP-13, as well as MMP-8 and MMP-2, which also participate in collagen breakdown.[1] The balance of the two effects can explain why QA-DC, while still inhibiting collagen degradation, is less effective than DC. Combination of the effects against MMP production and activity can also explain why QA-DC can antagonize collagen degradation while being inactive in the assays of proteoglycan breakdown.

3. Conclusion

The inhibition of collagen degradation by QA-DC is a promising result for the development of a selective chondroprotective agent for the treatment of osteoarthritis. Moreover, since we have previously shown in vivo that QA derivatives exhibited an increased affinity for cartilage,[15,16] we can hypothesize that the QA conjugates display a similar profile and preferentially concentrate in the chondral matrix. In addition, it is already known that quaternization of the C-4 dimethylamino group of tetracyclines results in the loss of their antibiotic properties.[29] These developments should allow a significant decrease of the efficient dose administered and hence an attenuation of adverse side effects. Future studies

have been planned in order to investigate the biodistribution of ^{14}C-labeled QA-TC and QA-DC, and confirm that these compounds can effectively target cartilage.

4. Experimental

4.1. General methods

All chemicals were obtained from commercial suppliers. Proton nuclear magnetic resonance (^1H NMR) and carbon nuclear magnetic resonance (^{13}C NMR) spectra were recorded on a Brüker AM 500 spectrometer at 500 and 50 MHz, respectively, and autocalibrated to the deuterated solvent reference peak. Chemical shift values (δ) are quoted in parts per million (ppm), and coupled constants (J) in hertz (Hz). Infrared spectra (IR) were recorded as KBr disks on a Brüker Vector 22 FTIR system. Electrospray ionization mass spectra (ESI-MS) were performed on a Brüker ESQUIRE-LC spectrometer. Melting points, uncorrected, were taken in glass capillary tubes on an Electrothermal digital melting point apparatus.

4.2. 1,4,4a,5,5a,6,11,12a-Octahydro-2-aminocarbo-nyl-3,6,10,12,12a-hexahydroxy-6-methyl-1,11-dioxo-naphthacene-4-trimethylammonium iodide (QA-TC)

Tetracycline hydrochloride dissolved in water was neutralized by NaOH (1 N) and the resulting precipitate was filtered and dried. Methyl iodide (1.36 mL, 21.83 mmol) was added under argon to a stirred solution of tetracycline (388 mg, 0.87 mmol) in anhydrous tetrahydrofuran (10 mL). The mixture was left at room temperature, and a yellow precipitate of QA-TC appeared within a few hours. The reaction was completed after 4 days. The residue was filtered, washed with water and ether, and dried under vacuum to afford QA-TC as a yellow solid (291 mg, 57%). Mp 212–214 °C (dec); IR (KBr) ν (cm^{-1}) 3500–2500 (NH_2, OH); ^1H NMR (DMSO-d_6): δ 1.50 (s, 3H, CH_3), 1.72 (br q, J = 12.0, 1H, H-5β), 2.25 (br d, J = 12.1, 1H, H-5α), 2.88 (dd, J = 4.6, J = 10.9, 1H, H-5a), 3.08 (d, J = 12.5, 1H, H-4a), 3.40 (s, 9H, $N^+(CH_3)_3$), 4.40 (s, 1H, H-4), 5.02 (s, 1H, OH-6), 6.91 (d, J = 8.3, 1H, H-9), 7.12 (d, J = 8.3, 1H, H-7), 7.55 (t, J = 8.3, 1H, H-8), 7.71 (s, 1H, OH-10), 9.43 (se, 1H, NH_2), 9.51 (se, 1H, NH_2), 11.74 (s, 1H, OH-3), 15.24 (se, 1H, OH-12); ^{13}C NMR (DMSO-d_6): δ 23.36 (CH_3), 27.74 (C-5), 36.84 (C-4a), 46.72 (C-5a), 54.75 ($N^+(CH_3)_3$), 68.79 (C-6), 73.09 (C-12a), 76.44 (C-4), 98.30 (C-2), 107.45 (C-11a), 115.32, 116.21, 118.02, 137.56, 148.73, 162.29 (aromatic carbons), 173.60 (C=O amide), 187.03, 192.96, 193.79 (C-1, C-3, C-11); ESI-MS m/z 459 $(M-I)^+$; elemental analysis: Calcd for $C_{23}H_{27}N_2O_8I{\cdot}2H_2O$, C, 44.38; H, 5.02; N, 4.50. Found: C, 44.77; H, 4.87; N, 4.49.

4.3. 1,4,4a,5,5a,6,11,12a-Octahydro-2-aminocarbo-nyl-3,5,10,12,12a-hexahydroxy-6-methyl-1,11-dioxo-naphthacen-4-trimethylammonium iodide (QA-DC)

Doxycycline hydrochloride dissolved in water was neutralized by NaOH (1 N) and the resulting precipitate was filtered and dried. Methyl iodide (350 μL, 5.63 mmol) was added under argon to a stirred solution of doxycycline (100 mg, 0.22 mmol) in anhydrous tetrahydrofuran (10 mL). The mixture was left at room temperature, and a brown precipitate of QA-DC appeared within a few hours. The reaction was completed after 24 h. The residue was filtered, washed with water and ether, and dried under vacuum to afford QA-DC as a brown solid (95 mg, 72%). Mp 204–206 °C (dec); IR (KBr) ν (cm^{-1}) 3500–2500 (NH_2, OH); ^1H NMR (DMSO-d_6) δ 1.44 (d, J = 5.2, 3H, CH_3), 2.48 (m, 1H, H-5a), 2.62 (m, 1H, H-4a), 3.10 (m, 1H, H-6), 3.35 (s, 9H, $N^+(CH_3)_3$), 4.55 (s, 1H, H-4), 5.69 (d, J = 10.4, 1H, OH-5), 6.85 (d, J = 7.9, 1H, H-9), 6.89 (d, J = 7.9, 1H, H-7), 7.53 (t, J = 7.9, 1H, H-8), 7.67 (s, 1H, OH-10), 9.09 (se, 1H, NH_2), 9.22 (se, 1H, NH_2), 11.45 (s, 1H, OH-3), 15.26 (se, 1H, OH-12); ^{13}C NMR (DMSO-d_6) δ 16.87 (CH_3), 39.54 (C-6), 44.33 (C-5a), 46.56 (C-4a), 55.56 ($N^+(CH_3)_3$), 69.10 (C-5), 73.14 (C-12a), 74.92 (C-4), 98.59 (C-2), 107.59 (C-11a), 116.31, 116.58, 116.81, 137.62, 148.66, 161.92 (aromatic carbons), 173.00 (C=O amide), 186.90, 192.89, 193.11 (C-1, C-3, C-11); ESI-MS m/z 459 $(M-I)^+$; elemental analysis: Calcd for $C_{23}H_{27}N_2O_8I{\cdot}H_2O$, C, 45.70; H, 4.84; N, 4.63. Found: C, 45.83; H, 4.96; N, 4.37.

4.4. Inhibition of MMP activity

The test was adapted from Chollet et al.[30] Reference compound AG3340 (prinomastat), a broad-spectrum MMP inhibitor,[31] was synthesized at the Institut de Recherches Servier. Human pro-MMPs were dissolved in developing buffer (Novex, Cat. No. LC2671) at the following concentrations: pro-MMP-2 (Boehringer) at 300 μg/mL; pro-MMP-3 (AbCys) and pro-MMP-8 (Pr. G. Murphy, Univ. East Anglia) at 1 μg/mL; pro-MMP-13 (Univ. East Anglia) at 2 μg/mL. Pro-enzymes were activated by 2 mM p-aminophenyl mercuric acetate (APMA, Sigma) at 37 °C for 30 min (MMP-2) or 1 h (MMP-3, -8, -13). Activation was stopped by transferring the samples to ice. Tetracyclines and derived molecules were dissolved at 10^{-2} M in dimethylsulfoxide (DMSO), then serially diluted (1/10) in developing buffer at concentrations ranging from 10^{-3} to 10^{-6} M. The fluorogenic substrate (Bachem) for MMP-3 was (7-methoxycoumarine-4-yl)-Arg-Pro-Lys-Pro-Tyr-Ala-Nva-Trp-Met-Lys(Dnp)-NH_2.[32] The fluorogenic substrate (Bachem) for MMP-2, -8, and -13 was Dnp-Pro-Cha-Gly-Cys(ME)-His-Ala-Lys(Nma)-NH_2.[33] These substrates were dissolved in DMSO at 10^{-2} and 2×10^{-3} M, respectively, and then diluted to 2×10^{-4} M in water. Assays were performed in 96-well plates by adding to each well 70 μL of developing buffer, 10 μL of inhibitor (or buffer for the control), and 10 μL of enzyme (or buffer for the blank). Each point was run in triplicate. After a 30-min preincubation at 37 °C, 10 μL of substrate was added and the plates were incubated for 6 h at 37 °C. Reading was then performed using a fluorimeter at excitation and emission wavelengths of 340 and 440 nm, respectively. Substrate degradation in the presence of inhibitor at a given concentration was calculated as % fluorescence of control wells. IC_{50} on each enzyme was calculated using EXCEL software from 3 points in the central linear

range of fluorescence inhibition. Activity enhancement was calculated using the formula $(IC_{50}(TC) - IC_{50}(QA - TC))/IC_{50}(TC) \times 100$, and the same formula was used for QA-DC. According to this formula, an IC_{50} from, for example, 100 to 50 μM corresponds to a 100% gain in activity. Conversely, a change from 100 to 200 μM corresponds to a 50% decrease in activity.

4.5. Expression of MMP-3 and MMP-13 mRNAs by SW-1353 cells

Human chondrosarcoma SW-1353 cells (ATCC) were seeded in 6-well culture plates at a density of 2.5×10^5 cells/well/2 mL of Ham's F12 media (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 1% of a stock solution of 10 IU/mL penicillin plus 10 mg/mL streptomycin (PS, Invitrogen). The media were changed every 2–3 days until confluence was attained. Cells were then washed with Hanks' balanced salt solution (HBSS, Invitrogen), then incubated in serum-free Ham's F12 media supplemented with 0.1% bovine serum albumin (BSA, Sigma) and 1% PS. After 24 h, the cells were treated with vehicle (DMSO) or tetracyclines (solubilized in DMSO at 10^{-2} M) at a final concentration of 10^{-4} M. Thirty minutes later, vehicle (basal control) or 1 ng/mL recombinant murine (rm) IL-1β (Sigma) was added. After 24 h, the media were discarded and the mRNA was analyzed according to the previously published protocol.[34] Briefly, total RNA was extracted using the Rneasy Mini Kit (Qiagen) following the manufacturer's instructions. Five micrograms of total RNA was loaded onto a 1% agarose gel containing ethidium bromide in order to check for RNA integrity and loading. Then, 2.5 μg of total RNA was reverse-transcribed (RT) for 1 h at 37 °C, using oligo d(T)12-18 and superscript II, in a total volume of 20 μL. Two microliters of the RT reaction was used for each PCR run. Gene-specific oligonucleotide primers were designed from the reported cDNA sequences of human MMP-3[35] and MMP-13,[36] and 18S rRNA (Clontech) was used as a housekeeping gene. The amplification profile used 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min in a Gene Amp PCR system 2400 (Perkin-Elmer) using VENT polymerase (Biolabs). The PCR products were then separated by electrophoresis on a 1% agarose gel and photographed under UV light.

4.6. Production of MMP-13 by SW-1353 cells

SW-1353 cells were seeded in 48-well culture plates at a density of 1.5×10^4 cells/well/0.5 mL of Ham's F12 media supplemented with 10% FBS and 1% PS. The media were renewed every 2–3 days until cells were confluent. After three washes with HBSS, cells were incubated in serum-free media supplemented with 0.1% BSA and 1% PS. After 24 h, the cells were treated with vehicle (DMSO) or tetracyclines at concentrations ranging from 6×10^{-6} to 5×10^{-5} M. After 30 min, vehicle (basal control) or rmIL-1β (10 ng/mL) was added. After another 24 h, the media were collected and total secreted MMP-13 (pro-enzyme plus mature form) was assayed using the ELISA Biotrak kit (Amersham).

4.7. Cartilage degradation

The detailed protocols of proteoglycan and collagen degradation have been published previously.[37] The tests are based on the culture of fragments of rabbit articular cartilage, as outlined below.

4.7.1. Proteoglycan degradation. Fragments were first incubated in the presence of $^{35}SO_4$ in order to label neosynthesized proteoglycans, then washed to eliminate unbound radiolabel and used for either of the following two assays. (1) MMP-production-dependent degradation: radiolabeled fragments were transferred to 96-well plates, in the absence (control) or presence of 1 ng/mL rmIL-1β, and supplemented or not with tetracyclines. After 24 h, the media were discarded and the fragments were transferred to plates containing 5×10^{-4} M APMA as pro-MMP activator. After another 24 h, the fragments were collected and digested in 0.6 mg/mL papain. Radioactivity in the culture media and in the tissue digest was measured by liquid scintillation using a β-counter (Beckman). (2) Activated-MMP-dependent degradation: radiolabeled fragments were first batch-stimulated with 10 ng/mL rmIL-1β for 24 h, then transferred to 96-well plates, in the absence (control) or presence of 5×10^{-4} M APMA, and supplemented or not with tetracyclines. After another 24 h, the media and fragments were collected and processed as above for measurement of proteoglycan degradation. For both assays, proteoglycan degradation in each fragment was expressed as the percentage of released radioactivity using the following formula: degradation = media radioactivity/(media radioactivity + tissue radioactivity) × 100.

4.7.2. Collagen degradation. Fragments (not radiolabeled) were transferred to 96-well plates, in the absence (control) or presence of 10 ng/mL IL-1β plus 0.1 U/mL EACA- and Lys-free plasmin (Calbiochem), and supplemented or not with tetracyclines. After 48 h, the tissue fragments and the media were hydrolyzed in 6 M HCl at 110 °C for 12 h. Hydroxyproline (OH-Pro) in media and tissue hydrolysates was assayed by colorimetry.[38] Collagen degradation in each fragment was expressed as the percentage of OH-Pro released using the following formula: degradation = media OH-Pro/(media OH-Pro + tissue OH-Pro) × 100.

4.8. Statistics

Data are expressed as means ± standard error of the mean (sem). Control and treated groups were statistically compared by analysis of variance followed by Dunnett's *t* test. Significance levels are shown as follows: $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$.

Acknowledgments

We thank Dr. Guillaume De Nanteuil (Division of Medicinal Chemistry, Institut de Recherches Servier)

for synthesis of AG3340. This work was supported by ADIR, Servier (France).

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