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RESEARCH PAPER

A novel non-antibacterial, non-chelating hydroxypyrazoline derivative of minocycline inhibits nociception and oedema in mice

LFS Bastos[1,4], A Angusti[2,4], MC Vilaça[1], LA Merlo[1], EB Nascimento Jr[1], LTS Rocha[1], AM Godin[1], AGR Solano[1], S Jarussophon[2,3], EA Nunan[1], Y Konishi[2] and MM Coelho[1]

[1]*Departamento de Produtos Farmacêuticos, Faculdade de Farmácia, Universidade Federal de Minas Gerais, Avenida Antônio Carlos 6627, Belo Horizonte, Minas Gerais, Brasil;* [2]*National Research Council Canada, Biotechnology Research Institute, Montréal, Quebec, Canada and* [3]*National Nanotechnology Center, National Science and Technology Development Agency, Klong Luang, Pathumthani, Bangkok, Thailand*

Background and purpose: Many *in vitro* and fewer *in vivo* studies have shown that tetracyclines present anti-inflammatory activity. We investigated if a novel non-antibacterial, non-chelating hydroxypyrazoline derivative of minocycline, 12*S*-hydroxy-1,12-pyrazolinominocycline (PMIN), also induced antinociceptive and anti-inflammatory effects.
Experimental approach: Antibacterial effects against a minocycline-sensitive *Staphylococcus aureus* strain were evaluated by applying a cylinder-plate agar diffusion technique. Antibacterial effects of diluted serum from mice pre-treated with minocycline or PMIN were also evaluated. Ca^{2+} binding activity was assessed by spectrophotometry. Formalin-induced nociceptive responses and carrageenan-induced paw oedema were evaluated in mice. The rota-rod apparatus was used to evaluate motor coordination.
Key results: Minocycline, but not PMIN, inhibited bacterial growth. Serum from mice treated with minocycline, but not with PMIN, also induced such an effect. The UV absorption spectrum of solutions of minocycline, but not those of PMIN, was markedly changed in the presence of Ca^{2+}. Minocycline or PMIN inhibited both phases of formalin-induced nociception and carrageenan-induced paw oedema. It is unlikely that antinociception resulted from lack of motor coordination, as tetracycline did not impair the performance of mice on the rotating rod.
Conclusions and implications: These results indicate that inhibition of nociception and oedema by tetracyclines is neither necessarily linked to antibacterial nor to Ca^{2+} chelating activities. This study supports the evaluation of the potential usefulness of PMIN in the treatment of painful and inflammatory diseases, as its lack of antibacterial and Ca^{2+} chelating activities might confer greater safety over conventional tetracyclines.
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Keywords: minocycline; 12*S*-hydroxy-1,12-pyrazolinominocycline; chemically modified tetracyclines; cylinder-plate assay; chelating activity; nociception; pain; oedema; inflammation

Abbreviations: ATCC, American Type Culture Collection; CMT, chemically modified tetracycline; IL, interleukin; PBS, phosphate-buffered saline; PMIN, 12*S*-hydroxy-1,12-pyrazolinominocycline; TNF-α, tumour-necrosis factor-α

Introduction

Tetracyclines, broad-spectrum bacteriostatic agents, may also be useful in the treatment of pathological conditions in which acute or chronic inflammation is involved, such as rheumatoid arthritis and neurodegenerative, dermatological and neoplastic diseases. A meta-analysis that evaluated the effectiveness of tetracyclines in the treatment of rheumatoid arthritis showed that these drugs, particularly minocycline, reduced disease activity (Stone *et al*., 2003). Many experimental studies have provided support to the potential benefit of tetracyclines in the treatment of inflammatory conditions. Recently, we demonstrated that doxycycline and minocycline, two second-generation semi-synthetic tetracyclines, inhibited inflammatory pain and oedema, fever, cell migration and formation of fibrovascular tissue in experimental animals (Bastos *et al*., 2007). The benefit of

Correspondence: Dr MM Coelho, Departamento de Produtos Farmacêuticos, Faculdade de Farmácia, Universidade Federal de Minas Gerais, Avenida Antônio Carlos, 6627, Belo Horizonte, Minas Gerais 31270-901, Brasil.
E-mail: mmcoelho@farmacia.ufmg.br
[4]These authors contributed equally to this work.
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minocycline in preventing diabetic retinopathy (Krady *et al*., 2005) and lesions induced by focal (Yrjänheikki *et al*., 1999) or global (Yrjänheikki *et al*., 1998) cerebral ischaemia have also been demonstrated in experimental studies. Minocycline is also a well-established inhibitor of microglial activation, an effect that may contribute to its antinociceptive activity, as these cells release several mediators, including cytokines and eicosanoids, which enhance synaptic transmission in the CNS (Watkins and Maier, 2003). The demonstration of these activities in the CNS has led to clinical trials to evaluate the usefulness of minocycline in the treatment of Huntington's or Parkinson's diseases, amyotrophic lateral or multiple sclerosis and mental dysfunction associated with human immunodeficiency virus infection (www.clinicaltrials.gov).

The chemically modified tetracyclines (CMTs), devoid of antibacterial activity, also attenuate some manifestations of the inflammatory response (Patel *et al*., 1999; D'Agostino *et al*., 2001; Sandler *et al*., 2005). Furthermore, some CMTs induce apoptosis in multidrug- and apoptosis-resistant leukaemia cell lines, and these effects underlie their potential antitumour activity (Tolomeo *et al*., 2001). Indeed, the efficacy and safety of incyclinide, also known as CMT-3, have been evaluated in clinical trials for the treatment of acne and some types of cancer (www.clinicaltrials.gov). The potential advantage of CMTs is that they would neither affect the microflora nor lead to the development of antibiotic-resistant microorganisms during prolonged use. Novel minocycline derivatives have been synthesized through its reaction with hydrazine, which yields both 1,12- and 11,12-substituted hydroxypyrazoline derivatives. Such a modification results in non-antibacterial, non-chelating 'pentacyclines' (for example, CMT-5), as substitutions at positions 1, 11 and 12 are invariably detrimental to antibacterial activity. Indeed, Lertvorachon *et al*. (2005) showed that 12*S*-hydroxy-1,12-pyrazolinominocycline (PMIN, Figure 1b) neither inhibits the growth of two minocycline-sensitive bacterial strains nor presents Zn^{2+} chelating activity.

It is still not clear whether the pleiotropic activities of tetracyclines in the experimental models of pain and inflammation result from the interaction with specific molecular target(s) or are dependent on a non-specific effect by chelating divalent ions, particularly Ca^{2+} and Zn^{2+}. Such a mechanism is not unlikely, as Ca^{2+} controls many cellular functions by acting ubiquitously as an intracellular second messenger as well as an extracellular first messenger (Brown *et al*., 1993; Prado, 2001). In addition, Zn^{2+} is a cofactor of matrix metalloproteinases, whose role in physiological processes and inflammatory and vascular diseases, such as atherosclerosis, angiogenesis, oxidative stress and ischaemia/reperfusion injury, has been under intense investigation (Chow *et al*., 2007; Hu *et al*., 2007).

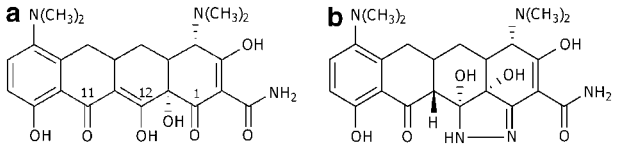


Figure 1 Structures of minocycline (**a**; the positions 1, 11 and 12 of the tetracyclic ring are shown) and 12*S*-hydroxy-1,12-pyrazolinominocycline (PMIN; **b**).

Thus, PMIN appeared to be a useful pharmacological tool to investigate whether the pleiotropic effects induced by tetracyclines are dependent on a non-specific effect by chelating divalent cations. It was also relevant to evaluate whether PMIN is converted into an antibacterial compound *in vivo*, as this aspect remained to be elucidated, and finally whether PMIN shares with minocycline, any antinociceptive and anti-inflammatory activities.

Materials and methods

Preparation of standard solutions and test samples for the microbiological assay

Stock solutions of minocycline and PMIN ($10\,mg\,L^{-1}$) were prepared in pH 4.5 phosphate-buffered saline (PBS) in amber glassware. Aliquots of the stock solutions were diluted in BSA (Sigma, St Louis, MO, USA) solution (2.5% $w\,v^{-1}$, pH 7.4) to obtain final solutions with minocycline concentrations of 53, 80, 120, 180 and $270\,\mu g\,L^{-1}$ (1.5 geometric progression). PMIN was tested at only one concentration ($270\,\mu g\,L^{-1}$). Three pools of serum from mice ($n=6$ per group) pre-treated (3 or 6 h) through the i.p. route with saline or equimolar doses of minocycline ($100\,mg\,kg^{-1}$) or PMIN ($95\,mg\,kg^{-1}$) were prepared and then diluted (240-fold) in the BSA solution. Lower dilutions (120- and 60-fold) of the pool from mice treated with PMIN were also prepared.

Bacteria culture

The American Type Culture Collection (ATCC, Manassas, VA, USA) originally supplied the *Staphylococcus aureus* ATCC 6538 bacterial strain, which was cultivated on antibiotic medium number 1 agar (BD Difco, Sparks, MD, USA) and incubated for 24 h at 35 °C. A spectrophotometer (Coleman 6/20, Maywood, NJ, USA) was used to monitor the optical densities of bacterial cultures. Diluted culture suspensions of 75 ± 1% absorbance (580 nm) were obtained. An aliquot of this suspension was mixed and then added into melted, tempered (48–50 °C in water bath) antibiotic medium number 8 to obtain a final concentration of 0.1% $v\,v^{-1}$. The medium number 8 resulted from the adjustment of the pH of the antibiotic medium number 2 (BD Difco, USA) to 5.6. The medium consisted of two layers. The liquefied antibiotic medium number 1 (6 mL) was poured into 100×20 mm Petri dishes to form a uniform layer. Immediately after hardening this base layer, 4 mL of inoculated antibiotic medium number 8 was poured onto each dish.

Cylinder-plate microbiological assay

Six sterile stainless steel cylinders ($8 \times 6 \times 10$ mm) were placed on the surface of the agar. Three alternated cylinders were filled with 200 µL of standard reference solution ($120\,\mu g\,L^{-1}$ of minocycline) and the other three with the remaining reference solutions or the test samples. A period of diffusion (1 h, 16 °C) prior to incubation was used. Clearly

defined zones of inhibition were produced after incubation (36.5 °C, 17 h) and then the diameters were measured using a digital calliper (Mitutoyo, Tokyo, Japan, resolution 0.01 mm). A zone of inhibition equal or more than the diameter of the cylinder (8 mm) was considered indicative of antibacterial effect. A correction factor was applied to compensate for between-plate variations.

These procedures were based on the microbiological assay for chlortetracycline determination (Grove and Randall, 1955) and on methods described in the British Pharmacopoeia (2007).

Ca^{2+} binding assay

In a 1 cm quartz cuvette, 0.1 mL solution of minocycline or PMIN (1 mM in water) was mixed with 0.8 mL methanol and 0.1 mL of 0.05 M Tris buffer (pH 7.4). The UV absorption spectrum of the solutions was obtained initially in the absence of Ca^{2+}. Next, an aliquot (10 μL) of an aqueous solution containing $CaCl_2$ (Anachemia, Lachine, Canada) was added to each solution and new absorption curves in the presence of Ca^{+2} (0.1, 0.5 or 1 mM) were obtained. The change in volume resulting from the addition of 10 μL $CaCl_2$ solution to 1 mL sample was ignored. The baseline absorption spectrum was recorded with 0.1 mL water, 0.8 mL methanol and 0.1 mL 0.05 M Tris buffer (pH 7.4) and was compared with the absorption spectra of the samples. The changes in the UV profile in the range of 200–500 nm region and the shift of the maximum absorption after addition of $CaCl_2$ were recorded by using a Varian (Cary 3E) UV-vis spectrophotometer.

Animals

All experiments were performed according to the ethical guidelines for the investigation of experimental pain in conscious animals (Zimmermann, 1983) and approved by the Ethics Committee on Animal Experimentation of the Federal University of Minas Gerais (Brazil) and by the Biotechnology Research Institute Animal Care Committee, which functions in accordance with the regulations of the Canadian Council of Animal Care. Male Swiss mice (20–25 g) were used. The animals had free access to food (antibiotic-free chow) and water and were maintained in a room with a 12 h light–dark cycle for at least 3 days before the experiment to allow for acclimatization. The experiments were conducted at room temperature between 27 and 28 °C, which corresponds to the thermoneutral zone for rodents (Gordon, 1990).

Evaluation of the nociceptive response induced by formalin in mice

Formalin (2.5% v v^{-1}, 20 μL) was injected through the s.c. route into the dorsum of the right hind paw of mice 1 h after the i.p. administration of minocycline or PMIN. Each mouse was placed under a transparent glass funnel (18 cm diameter, 15 cm high) and the amount of time the animal intermittently licked the injected paw was measured between 0 and 5 min (first phase) and 15 and 30 min (second phase) after the injection of formalin.

Evaluation of the motor coordination of mice in the rota-rod

The motor coordination of the animals was evaluated in a rota-rod apparatus. The animals were trained on the apparatus for 3 days before the experiment. On the experimental day, the animals were placed on the rotating rod (12 r.p.m.) and the time they spent on it was measured. The cutoff time was 60 s. After confirming that all animals were able to stay on the rotating rod for at least 60 s, they were treated with the tetracycline derivatives or phenobarbital (positive control) and 1 h later they were again tested on the apparatus.

Evaluation of the paw oedema induced by carrageenan in mice

Paw oedema was measured using a plethysmometer (Model 7140, Ugo Basile, Comerio, Italy). The basal volume of the right hind paw was determined before administration of any drug. After determination of the basal volume, the animals were divided in the experimental groups in such a way that the mean volumes of the different groups were similar. Minocycline or PMIN were administered 1 h before intraplantar injection of carrageenan (300 μg, 20 μL). The paw volume was measured at different times after injection of the inflammatory stimulus. The results are presented as the difference in paw volume, from the basal values.

Data analysis

After obtaining a corrected standard curve, a linear regression was calculated for the antibacterial activity assay. A correction factor was applied to compensate for between-plate variations. Each antibiotic dilution value, except for the standard reference (intermediate concentration), was calculated from a set of three plates. Average zones of all standard reference concentrations on the entire set of plates were calculated. For each set of three plates, the measures of the nine standard reference zones and the nine measures of the other concentrations used for the standard curve were averaged. Then the nine-zone average of the standard reference was subtracted from the cumulative standard reference average and finally the resulting value was algebraically added to the value of the average zone measure for the antibiotic concentration used on that set of plates. The standard curve was calculated on a semilogarithmic graph (using the corrected values) with the zone diameters on the arithmetic scale and the antibiotic concentrations on the logarithmic scale. The linear regression from the standard curve was used for calculating values in quantitative analysis of test samples.

The results from the tests with experimental animals, presented as mean ± s.e.mean, were analysed by one-way ANOVA followed by Newman–Keuls *post hoc* test for multiple comparisons. Values of $P<0.05$ were considered to show significant differences between means.

Drugs

12*S*-hydroxy-1,12-pyrazolinominocycline (PMIN) was synthesized as previously described by Lertvorachon *et al.* (2005). Minocycline hydrochloride (Galena, Campinas, Brazil),

λ-carrageenan type IV (Sigma, USA), formaldehyde 40% m v^{-1} (Carlo Erba, Rodano, Italy) and phenobarbital (Aventis Pharma, São Paulo, Brazil) were also used. Solutions and suspensions were prepared in isotonic saline immediately before the injections. The volume of i.p. injection was 10 mL kg^{-1}.

A standard pharmacological nomenclature, which conforms to the *British Journal of Pharmacology*'s Guide to Receptors & Channels (Alexander *et al*., 2008) was used throughout.

Results

PMIN does not inhibit the growth of S. aureus

A standard curve of growth inhibition of a minocycline-sensitive *S. aureus* strain showed that minocycline inhibited the bacterial growth in a concentration-dependent manner (Figure 2). The correlation coefficient was $r = 0.9980$ and the equation was $y = 12.98 \log(x) + 24.92$. This curve was reproduced with statistically similar results. The diluted (240-fold) serum from mice pre-treated with minocycline also produced well-defined zones of growth inhibition. By applying the equation, it was possible to estimate the serum concentrations of minocycline (and/or its putative product(s) of biotransformation with antibacterial activity), which were 32.9 and 21.4 mg L^{-1} at the third and sixth hours, respectively, after i.p. injection of MIN. On the other hand, the diameters of inhibition zones of bacterial growth measured after adding either a standard solution of PMIN (270 μg L^{-1}) or saline to bacterial cultures, were smaller than 8 mm (the diameter of the cylinder). The diluted serum (240-, 120- and 60-fold) from mice pre-treated with PMIN (95 mg kg^{-1}) or saline also yielded a diameter of inhibition zones smaller than 8 mm.

PMIN does not present Ca^{2+} chelating activity

The Ca^{2+} binding activity of the tetracycline derivatives was investigated. As expected, the UV absorption spectrum of solutions of minocycline (1 mM) was markedly changed after the addition of $CaCl_2$ to provide different concentrations of Ca^{2+} (0.1, 0.5 and 1 mM, Figure 3a). Under the same conditions, minor shifts in the absorption coefficient and no shift in maximal λ were observed for PMIN (Figure 3b).

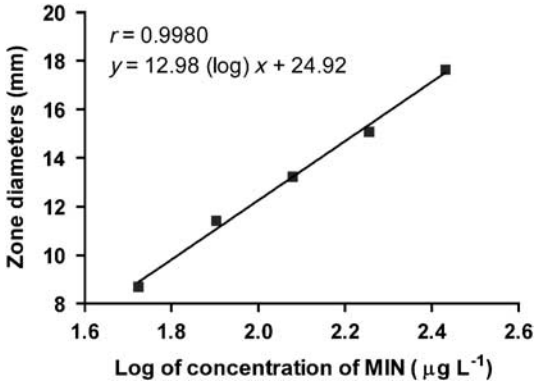


Figure 2 Effect induced by minocycline (MIN; 53, 80, 120, 180 and 270 μg L^{-1} in pH 7.4 PBS containing BSA) on the growth of *Staphylococcus aureus* ATCC 6538 in a cylinder plate-based assay. The correlation coefficient and the equation are shown. A correction factor was applied to compensate for between-plate variations. Data are expressed as mean ± s.e.mean of nine replicates, except the intermediate concentration (standard reference), which corresponds to 36 replicates (see 'Materials and methods' for details).

PMIN inhibits both phases of formalin-induced nociceptive response

After demonstrating the marked differences between minocycline and PMIN in the previous assays, their activities in two experimental models of pain and inflammation were investigated. The s.c. injection of formalin (2.5%, 20 μL) in mice induced an immediate nociceptive response characterized mainly by licking the injected paw. Figure 4 shows that the first (0–5 min) and the second (15–30 min) phases of this response were inhibited by previous (1 h) i.p. treatment with either minocycline (100 mg kg^{-1}) or PMIN (95 mg kg^{-1}).

PMIN does not impair motor coordination

The previously mentioned treatments did not alter the time spent by mice on the rota-rod apparatus, evaluated during 60 s. However, a marked impairment of their performance was observed 1 h after i.p. injection of phenobarbital (40 mg kg^{-1}), used as a positive control (Figure 5).

PMIN inhibits carrageenan-induced oedema

The intraplantar injection of carrageenan (300 μg, 20 μL) in mice induced oedema. The increase of paw volume was

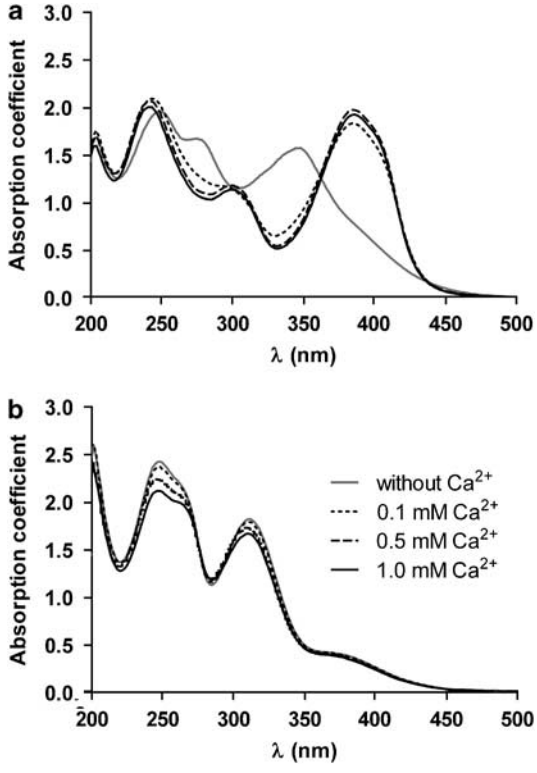


Figure 3 Ultraviolet spectra of minocycline (**a**; 1.0 mM) and 12*S*-hydroxy-1,12-pyrazolinominocycline (PMIN 1.0 mM; **b**) in the absence or in the presence of increasing concentrations of Ca^{2+} (0.1, 0.5 and 1.0 mM).

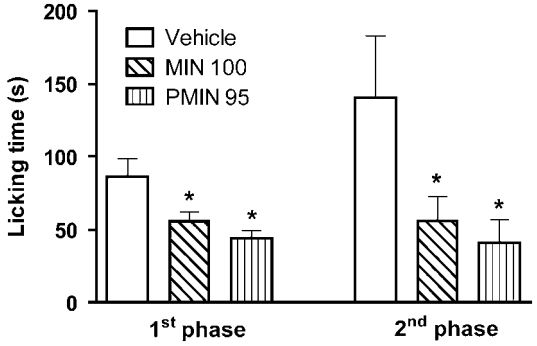


Figure 4 Effects induced by minocycline (MIN; 100 mg kg^{-1}, i.p.,-1 h) or 12*S*-hydroxy-1,12-pyrazolinominocycline (PMIN; 95 mg kg^{-1}, i.p.,-1 h) on the nociceptive response induced by formalin (2.5% v v^{-1}, 20 μL, s.c.) in mice. Data are expressed as mean ± s.e.mean. ($n=8$).*$P<0.05$ compared with the vehicle-treated group, tested by ANOVA followed by Newman–Keuls *post hoc* test.

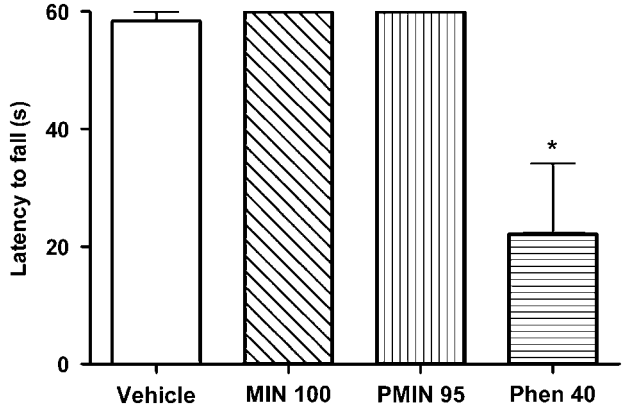
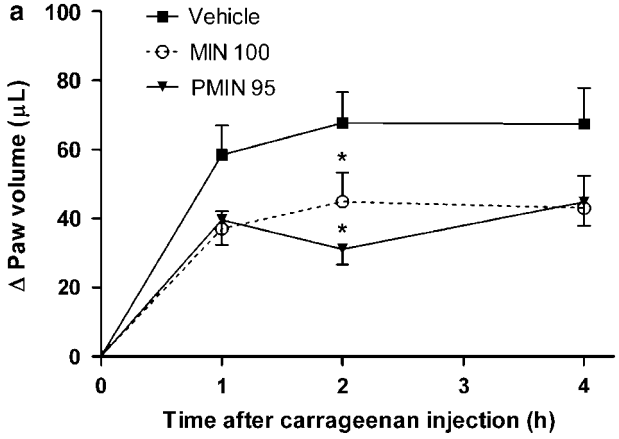


Figure 5 Effects induced by minocycline (MIN; 100 mg kg^{-1}, i.p.,-1 h), 12*S*-hydroxy-1,12-pyrazolinominocycline (PMIN; 95 mg kg^{-1}, i.p.,-1 h) or phenobarbital (Phen; 40 mg kg^{-1}, i.p.,-1 h) on the time spent by mice on the rotating rod (12 r.p.m., 60 s cutoff time). Data are expressed as mean ± s.e.mean. ($n=6$–7). *$P<0.05$ compared with the vehicle-treated group, tested by ANOVA followed by Newman–Keuls *post hoc* test.



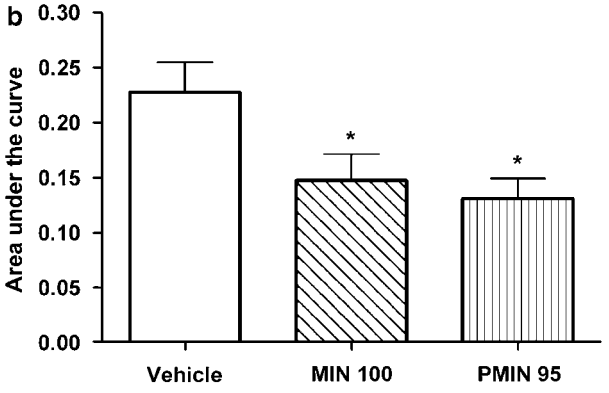


Figure 6 Effects induced by minocycline (MIN; 100 mg kg^{-1}, i.p.,-1 h) or 12*S*-hydroxy-1,12-pyrazolinominocycline (PMIN; 95 mg kg^{-1}, i.p.,-1 h) on the carrageenan-induced paw oedema (300 μg, 20 μL, intraplantar injection) in mice. Time course (**a**) and its respective area under the curve (**b**, arbitrary units) are shown. The mean basal paw volumes of the groups were: 86 ± 10, 92 ± 10 and 83 ± 10 μL, respectively. Data are expressed as mean ± s.e.mean. ($n=9$–10). *$P<0.05$ compared with the vehicle-treated group, tested by ANOVA followed by Newman–Keuls *post hoc* test.

evident 1 h after injection of carrageenan, reached its peak at the second hour and lasted at least 4 h (Figure 6a). Previous (1 h) treatment with minocycline (100 mg kg^{-1}) or PMIN (95 mg kg^{-1}) inhibited the oedema. The extent of inhibition up to the 4th h was about 40%, as can be observed by analysing the area under the curve (Figure 6b).

Discussion and conclusion

The present study showed that PMIN, a novel non-antibacterial, non-chelating hydroxypyrazoline derivative of minocycline, as well as the parent tetracycline, induced antinociceptive and antioedema effects in mice. These results represent the first demonstration of the antinociceptive effect induced by a member of the CMT class and also provide support to the observations that these compounds exhibit anti-inflammatory activity.

First, we developed a reliable and inexpensive *in vitro* method to evaluate the antibacterial activity of new tetracycline derivatives. It was possible to analyse both standard solutions and biological samples. As expected, minocycline markedly inhibited the growth of *S. aureus* in a concentration-dependent manner, whereas PMIN at a concentration of 270 μg L^{-1} did not induce such an effect. Regarding the broad spectrum of tetracyclines, further studies to test the activity of PMIN against various bacterial strains are necessary to reinforce the possibility of complete lack of antibacterial effect. However, the non-antibacterial profile of PMIN is supported by the demonstration that even at the high concentration of 60 mg L^{-1} this tetracycline derivative did not inhibit the growth of two minocycline-sensitive *Escherichia coli* strains (Lertvorachon *et al*., 2005).

By testing the effect induced by samples of serum obtained from animals treated with the tetracycline derivatives, it was also possible to evaluate the antibacterial effect *in vivo*. This is particularly important when evaluating the activity of PMIN, as the potential cleavage of the pyrazoline ring, a process that is not highly unlikely, as it has been demonstrated for other drugs (Bazin *et al*., 2007), might lead to the

formation of corresponding tetracyclines with antibacterial activity. If such a process occurred *in vivo*, the potential advantage of PMIN over usual tetracycline derivatives in the treatment of non-infectious conditions would be lost. As expected, the diluted serum from mice pre-treated with minocycline exhibited antibacterial activity, and the estimated concentrations of minocycline (20 to 30 $\mu g\,mL^{-1}$) were in the range of those shown by Fagan *et al*. (2004) after treating rats with 90 $mg\,kg^{-1}$ of MIN. In contrast, the diluted serum from mice pre-treated with PMIN did not present antibacterial activity. This result indicates that it is unlikely that PMIN, at least until 6 h after a single i.p. administration, is converted *in vivo* into tetracycline derivatives with antibacterial activity.

Besides the lack of antibacterial activity, PMIN was shown not to chelate Ca^{2+}. These results are compatible with the observations that substitutions at positions 1 and 12 of the minocycline molecule usually result in non-chelating derivatives. The lack of this activity may also impair the bacteriostatic effect, as tetracyclines inhibit protein synthesis in bacteria by a mechanism that involves Mg^{2+} chelation in the ribosomes (White and Cantor, 1971). The interest in PMIN derives in part from the possibility that it might neither cause candidiasis nor gastrointestinal disturbance, adverse effects associated with the prolonged use of the antibacterial minocycline (Goulden *et al*., 1996). In addition, as PMIN also lacks the Ca^{+2} chelating activity, another potential advantage over the usual tetracyclines would be the reduced possibility of deposition in the bones and induction of teeth staining because of the formation of a tetracycline-Ca^{+2} orthophosphate complex (Sánchez *et al*., 2004).

On the basis of our previous study (Bastos *et al*., 2007), a known effective dose of minocycline (100 $mg\,kg^{-1}$) and an equimolar dose of its derivative PMIN (95 $mg\,kg^{-1}$) were used to allow a comparison between activities presented by the two drugs in models of pain and inflammation with a minimal number of experimental animals. Both drugs inhibited the biphasic nociceptive response induced by formalin. It is unlikely that motor incoordination or muscle relaxation contributed to the reduced nociceptive behaviour of the animals treated with the tetracycline derivatives, as these drugs did not impair their performance in the rota-rod test. Similar results have also been observed for doxycycline in our previous study (Bastos *et al*., 2007). Moreover, both minocycline and PMIN inhibited the paw oedema induced by carrageenan in mice. These results indicate that the tetracycline derivatives also inhibit vascular changes and plasma leakage at the inflammatory site, events that contribute to the development of oedema.

As minocycline and PMIN inhibited the nociception induced by formalin and the paw oedema induced by carrageenan, it is important to briefly indicate the main events that contribute to these two responses before discussing possible mechanisms that may explain the activities of the tetracycline derivatives. Whereas the first phase of the nociceptive response induced by formalin is related to direct activation of TRPA1 receptors expressed in nociceptors (McNamara *et al*., 2007), the second phase is characterized mainly by local inflammation and central sensitization in the dorsal horn of the spinal cord (Tjølsen *et al*., 1992). The role played by inflammatory mediators, such as bradykinin, interleukin (IL)-1β, IL-6, IL-8 and tumour-necrosis factor-α (Chichorro *et al*., 2004), eicosanoids and nitric oxide (Hunskaar and Hole, 1987; Moore *et al*., 1991) in the second phase of the nociceptive response induced by formalin has been demonstrated. The oedema induced by carrageenan is also associated with the production of many inflammatory mediators, such as those previously mentioned, in addition to cell migration and activation of oxidative stress (Vinegar *et al*., 1987; Salvemini *et al*., 1996; Loram *et al*., 2007).

Many activities may explain the effects induced by minocycline in the two experimental models used in the present study. Taking into account that minocycline is a well-established inhibitor of microglial activation (Raghavendra *et al*., 2003; Ledeboer *et al*., 2005), this effect may explain, at least in part, its antinociceptive activity. In addition, other putative molecular targets may explain its antinociceptive and antioedema activities. Minocycline, directly or indirectly, prevents activation of many enzymes involved in the synthesis of multiple inflammatory mediators in microglia/macrophage or neuronal cell lines or in cell-free assays. Among them, $PL(A_2)$ (Pruzanski *et al*., 1992), different PK(C) isoforms (Webster *et al*., 1994; Nikodemova *et al*., 2007), multiple mitogen-activated protein kinases (Tikka *et al*., 2001; Nikodemova *et al*., 2006; Piao *et al*., 2006), phosphatidylinositol 3-kinase/Akt (Pi *et al*., 2004), inducible NOS (Amin *et al*., 1996), COX-2 (Kim *et al*., 2004), 5-lipoxygenase (Song *et al*., 2006) and poly(ADP-ribose) polymerase-1 (Alano *et al*., 2006).

The results of the present study provide further support for the observations that substitutions at positions 1 and 12 are invariably detrimental for the antibacterial activity of the tetracycline derivatives and also abolish their Ca^{+2} chelating activity. Indeed, there were marked differences between minocycline and PMIN in the microbiological and Ca^{+2} binding assays, nevertheless both drugs induced similar antinociceptive and antioedema effects. Thus, some structural changes, particularly the addition of a pyrazoline ring, seem not to reduce the antinociceptive and anti-inflammatory activities of tetracyclines. Considering the similar chemical structures and activities in the models of nociception and oedema, it is likely that minocycline and PMIN interact with similar targets to induce their antinociceptive and antioedema effects.

The mechanisms by which minocycline and others tetracyclines induce their pleiotropic effects remain to be clearly elucidated, but the present study provides strong evidence that their antinociceptive and antioedema activities may not be dependent on an non-specific chelation of divalent cations. Indeed, some *in vitro* studies have already investigated the putative involvement of Ca^{2+} in the anti-inflammatory activity of some tetracyclines. Gabler and Creamer (1991) showed that the suppression of neutrophil functions by tetracycline is only partially dependent on Ca^{2+} concentration. Moreover, the inhibition of enzymic activity of $PL(A_2)$ by minocycline is not reversed by excessive addition of Ca^{2+} (Pruzanski *et al*., 1992) and the prevention of neuronal apoptosis induced by glutamate is not

dependent on blockade of its ionotropic receptors or reduction of subsequent Ca^{2+} intracellular rises (Pi *et al.*, 2004).

In conclusion, the present study represents the first demonstration of the antinociceptive and antioedema effects induced by a CMT. Further studies are necessary to better characterize the pharmacological activities and the pharmacokinetic and toxicological profiles of PMIN. The comprehensive understanding of the activities and the structure–activity relationship of the CMTs may lead to the discovery of effective and safe drugs to be used in the treatment of different painful, inflammatory and neurodegenerative diseases.

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Conflict of interest

The authors state no conflict of interest.

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