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Trypanocidal structure–activity relationship for *cis*- and *trans*-methylpluviatolide

R. da Silva [a], J. Saraiva [b], S. de Albuquerque [b], C. Curti [c], P.M. Donate [d], T.N.C. Bianco [a], J.K. Bastos [c], M.L.A. Silva [a,*]

[a] *Núcleo de Ciências Exatas e Tecnológicas, Universidade de Franca, Avenida Dr. Armando Salles de Oliveira, 2001, 14404-600 Franca, SP, Brazil*
[b] *Departamento de Análises Clínicas, Toxicológicas e Bromatológicas da Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Avenida do Café, s/n, 14040-903 Ribeirão Preto, SP, Brazil*
[c] *Departamento de Ciências Farmacêuticas da Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Avenida do Café, s/n, 14040-903 Ribeirão Preto, SP, Brazil*
[d] *Departamento de Química da Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Avenida Bandeirantes, 3900, 14040-901 Ribeirão Preto, SP, Brazil*

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ABSTRACT

The trypanocidal activity of racemic mixtures of *cis*- and *trans*-methylpluviatolides was evaluated *in vitro* against trypomastigote forms of two strains of *Trypanosoma cruzi*, and in the enzymatic assay of *T. cruzi* gGAPDH. The cytotoxicity of the compounds was assessed by the MTT method using LLC-MK2 cells. The effect of the compounds on peroxide and NO production were also investigated. The mixture of the *trans* stereoisomers displayed trypanocidal activity (IC_{50} ~89.3 μM). Therefore, it was separated by chiral HPLC, furnishing the (+) and (−)-enantiomers. Only the (−)-enantiomer was active against the parasite (IC_{50} ~18.7 μM). Despite being inactive, the (+)-enantiomer acted as an antagonistic competitor. *Trans*-methylpluviatolide displayed low toxicity for LLC-MK_2 cells, with an IC_{50} of 6.53 mM. Furthermore, methylpluviatolide neither inhibited gGAPDH activity nor hindered peroxide and NO production at the evaluated concentrations.

1. Introduction

The lignan class of compounds presents a conspicuous chemical diversity, which varies substantially with respect to their enantiomeric composition. Natural lignans usually occur as either predominantly one enantiomer or as enantiomeric mixtures of various enantiomeric compounds (% e.e'. values) (Umezawa et al, 1997a).

In the last decade, the search for antiprotozoal compounds from natural sources has intensified (Rocha et al., 2005; Da Silva Filho et al., 2004; Caniato and Puricelli, 2003). This search has been mainly in plants, which continue to be a major source of biologically active metabolites that may provide lead compounds for the development of new drugs.

The enantiomeric properties of lignans can be summarized into four statements. First, dibenzylbutyrolactone lignans can be optically pure (>99% e.e.), while furofuran and furan lignans can be found as a mixture of both enantiomers of varying enantiomeric composition (Umezawa et al., 1997a). Secondly, most dibenzylbutyrolactone lignans are levorotatory, except for the lignans present in *Thymelaeaceae* plants and *Selaginella doederleinii,* which are dextrorotatory (Chen et al., 2001; Lin et al., 1994; Umezawa and Shimada, 1996; Umezawa et al., 1997a; Xu et al., 2001). Third, the predominant enantiomers of furofuran, furan, and dibenzylbutane lignans vary among plant species, and they differ even within the organs of a single plant species (*Arctium lappa*) (Umezawa et al., 1991; Umezawa et al., 1992; Umezawa et al., 1997b). Fourth, the absolute configurations of the predominant enantiomers of various lignans isolated from a single plant specimen are sometimes different.

In nature, it is possible to find two enantiomeric forms of the same lignan, but these forms bear different stereochemistry, depending on the plant species in which it occurs. In the meantime, only one of the lactone lignan enantiomers, (+)- or (−), has been found to occur in the same plant. Therefore, biological assays carried out with either plant extracts containing lignan lactones or its isolated lignans generally allow the evaluation of only one enantiomer, making it impossible to determine whether the other enantiomer displays activity.

Although lignan lactones are present in numerous plants used in popular medicine (Bianchi et al., 1968; Broomhead and Dewick, 1990; Cho et al., 1999), their trypanocidal activity against *Trypanosoma cruzi* has only recently been discovered by Bastos et al. (1999), who has investigated the trypanocidal activity of the *Zanthoxyllum naranjillo* lignans, and discovered one of the most powerful compounds regarding trypanocidal activity, (-)-methylpluviatolide (**1**) (Bastos et al., 1999; Silva et al., 2003), (Fig. 1).

* Corresponding author. Tel.: +55 16 3711 8871; fax: +55 16 3711 8878.
E-mail address: mlasilva@unifran.br (M.L.A. Silva).

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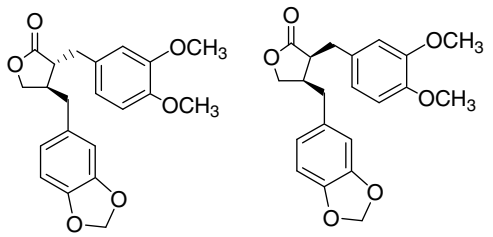


Fig. 1. Structural formulas of *trans*-methylpluviatolide (**1**) and *cis*-methylpluviatolide (**2**).

Lignans, one of the longest known classes of natural products, have attracted much interest over the years on account of their broad range of biological activities, such as antileishmanial (Barata et al., 2000), antimalarial (Zhang et al., 2001), antitumor (Bastos et al., 1996), and anti-inflammatory (Souza et al., 2004) activities, among others. Recently, our group reported the significant trypanocidal and antileishmanial activities of dibenzylbutyrolactone lignans (Souza et al., 2005; Royo et al., 2003; Bastos et al., 1999). Such results aroused the interest within our research group to study the effect of the stereochemistry and the absolute configuration of methylpluviatolide on its trypanocidal activity. To this end, methylpluviatolide was synthesized in its *trans* (**1**) and *cis* (**2**) racemic forms (Fig. 1), allowing us to evaluate the trypanocidal activity not only of a mixture of these two stereoisomers, but also of the pure enantiomers, which were separated by chiral HPLC.

2. Results and discussion

The results obtained against *T. cruzi* show that the racemic *cis*-stereoisomer (**2**) is inactive, while the racemic *trans*-stereoisomer (**1**) displays trypanocidal activity, with an IC_{50} ~89.3 μM. Furthermore, the latter stereoisomer (**1**) presents low toxicity for LLC-MK_2 cells, with an IC_{50} ~6.53 mM. (Fig. 2).

Our result is different from that obtained for the pure (−)-*trans*-methylpluviatolide (**1**) by Bastos et al. (1999), who reported 100% activity at a concentration of 50 μg and an IC_{50} value of 1.3 μg mL^{-1}. This result indicates that the *trans* isomer of this compound should display higher trypanocidal activity. However, it should be pointed out that the obtained results were undertaken for the racemic mixture of *cis* and *trans*-methylpluviatolide isomers. The (+/−)-*cis* (**1**) displayed the following percentage of lysis: 8.0 μM (1.4 ± 1.2), 32.0 μM (8.7 ± 2.7), 128.0 μM (16.8 ± 3.9) and 256 μM (32.2 ± 4.7), with an IC_{50} (μM mL^{-1}) of 641.0. The (+/−)-*trans* (**2**) displayed the following percentage of lysis: 8.0 μM (24.6 ± 1.9), 32.0 μM (39.2 ± 1.0), 128.0 μM (55.0 ± 5.6) and 256 μM (67.9 ± 0.0), with an IC_{50} (μM mL^{-1}) of 89.3.

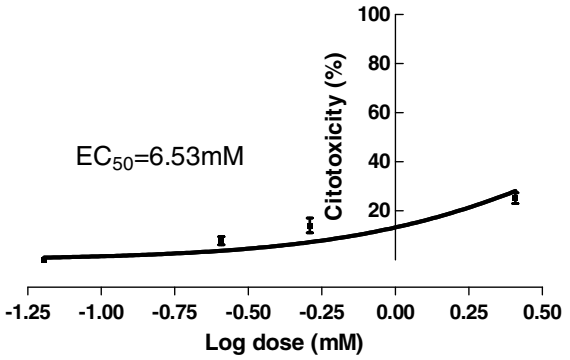


Fig. 2. Cytotoxic potential of (−)-methylpluviatolide (**1**) for LLC-MK2 cells.

Although the concentrations used by Bastos et al. (1999) differed from ours, the fact that the racemic mixture of the *trans* stereoisomer evaluated this time displayed lower activity than the pure compound evaluated before might indicate that the (+)-*trans* enantiomer should compete for the active sites in the parasite and might act as an antagonist in relation to the (−)-*trans* enantiomer.

On the basis of these results, a separation of the *trans*-stereoisomer from the racemic mixture was undertaken by chiral HPLC using an analytical Chiracel OJ (4.6 × 250 mm) column, aiming to evaluate the trypanocidal activity of each enantiomer separately. The chromatogram gave a well resolved peak separation, allowing the isolation of both enantiomers. Therefore, it was possible to compare the trypanocidal activity of both isomers, since in our previous work only the (−) isomer from *Zanthoxylum narangillo* was isolated (Bastos et al., 1999). The results showed that the (+) – *trans*-methylpluviatolide (**1**) displayed the following percentage of lysis: 8.0 μM (5.3 ± 2.5), 32.0 μM (7.6 ± 3.4) and 128.0 μM (9.9 ± 2.5), with IC_{50} (μM mL^{-1}) of 1.3 × 10^6. The (−) – *trans*- methylpluviatolide (**1**) displayed the following percentage of lysis: 8.0 μM (40.6 ± 3.6), 32.0 μM (52.3 ± 4.3) and 128.0 μM (79.7 ± 0.0), with IC_{50} (μM mL^{-1}) of 18.7.

The results show that the (+)-enantiomer is completely inactive, whereas the (−)-enantiomer displayed good activity, with an IC_{50} (μM) = 18.7 and 79.7% lyses, at concentrations lower than those used by Bastos et al. (1999), which corresponded to 50 μg/mL, IC_{50} = 1.3 μg and 100% lyses. These results indicate that, despite being completely inactive, the (+)-enantiomer of (**1**) blocks the action of the (−)-enantiomer when they are present in a racemic mixture. It should be taken into consideration that the (+)-enantiomer might bind to the active sites as a competitive antagonist, which may be confirmed by comparison of the IC_{50} value (μM) of the racemic mixture with that of the (−)-enantiomer itself.

Some metabolic routes were evaluated, such as the methylpluviatolide mechanism of action. In this regard, trypanosomatids are highly dependent on glycolysis for ATP production (Opperdoes, 1987; Opperdoes and Michels, 2001). The enzyme glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) catalyzes the oxidative phosphorylation of D-glyceraldehyde-3-phosphate into 1,3-diphosphoglycerate in the presence of NAD^+ and inorganic phosphate, and can be one of the preferred steps for inhibition (Bakker et al., 1999; Bakker et al., 2000). However, the obtained results demonstrated that methylpluviatolide does not exert inhibitory activity on this enzyme, displaying 0% inhibition at 100 μM.

The effect of methylpluviatolide (**1**) on nitric oxide and H_2O_2 production is shown in Figs. 3 and 4, respectively. The trypanocidal activity of several types of natural and synthetic compounds has been partly attributed to reactive oxygen species generated by the reduction of drugs (Uchiyama et al., 2005). Regarding the obtained results, the production of NO by cells was significantly lower when treated with methylpluviatolide (Figs. 3 and 4). Likewise, the literature reports that benzonidazole, a drug used for the treatment of Chagas disease, also inhibits the production of NO by modifying the balance between pro and anti-inflammatory mediators, with important consequences for the development of the disease (Piaggio et al., 2001; Stoppani, 1999). Fig. 4 shows that methylpluviatolide displays a weak effect in enhancing the peroxide production by *T. cruzi*. Some drugs, such as nifurtimox and gentian violet, act in the parasite by increasing the production of free radicals (Raether and Hanel, 2003).

Moreover, *T. cruzi* is more sensitive to free radicals, as it has a deficient antioxidant mechanism in comparison with host cells. This is especially the case for hydrogen peroxide, as *T. cruzi* does not produce catalase, an antioxidant enzyme responsible for the conversion of hydrogen peroxide into water and molecular oxygen

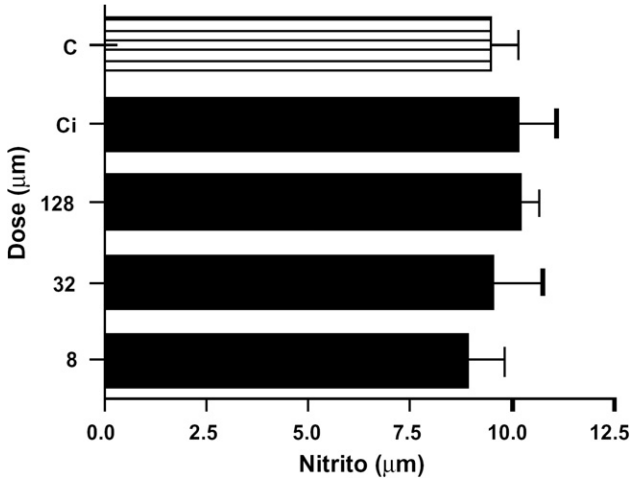


Fig. 3. Nitric oxide production of mouse peritoneal macrophages treated with methylpluviatolide (**1**). (C: cells control without *T. cruzi* infection; Ci: infected cells control).

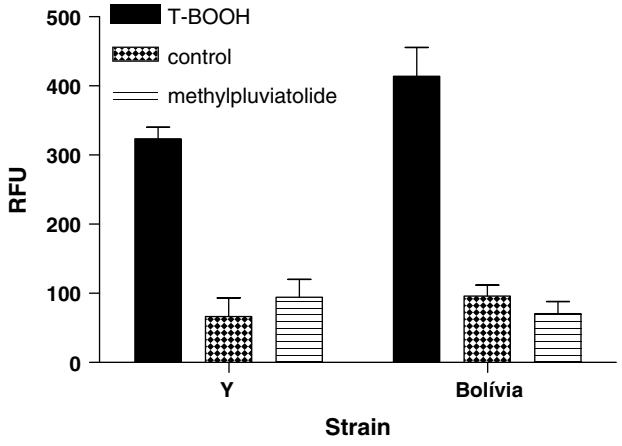


Fig. 4. Effects of methylpluviatolide (**1**) on H_2O_2 produced by *T. cruzi*. (RFU: relative fluorescence unit; T-BOOH: *t*-butyl hydroperoxide).

(Turrens and Mccord, 2006). Therefore, we could conclude that the trypanocidal mechanism of action of this class of compounds might not be related to the increase in the production of peroxides.

Recently, we have described the racemic 7′-oxi-methylpluviatolide, racemic 7′-hydroxi-methylpluviatolide and racemic 7′-oxi-6-nitro-methylpluviatolide as potential respiratory chain complex I inhibitors, a finding of possible relevance due to either the toxicological or therapeutical importance of these compounds (Saraiva et al., 2005).

Therefore, other metabolic routes should be evaluated to elucidate the mechanisms of action of (−)-methylpluviatolide.

3. Conclusion

In conclusion, it is possible to state that the *trans*-stereochemistry is the one responsible for the trypanocidal activity of methylpluviatolide (**1**), probably because it allows the correct binding of this compound to the active sites on the parasite, which is not possible with the *cis*-stereoisomer. As for the absolute configuration of the *trans*-stereoisomers, the inactive (+)-enantiomer probably acts as an antagonist, hindering the binding of the (−)-enantiomer to the active sites on the parasite, therefore decreasing the trypanocidal activity of methylpluviatolide when it is present as a racemic mixture.

4. Experimental

4.1. Reagents, solvents and compound identification procedures

Sulfanilamide, L-glutamine, tetrazolium salt, Triton x, $NaHCO_3$, 20,70-dichlorodihydrofluorescein diacetate (H_2DCFDA), *t*-butyl hydroperoxide and Hepes-KOH were purchased from Sigma–Aldrich Co., St. Louis, MO, USA. Dimethyl sulfoxide (DMSO) isopropanol and sucrose were purchased from Merck Co., whereas RPMI-1640 and fetal calf serum were obtained from GIBCO. Penicillin and streptomycin were acquired from Invitrogen. Optical rotations were measured at $k = 589$ nm on a Schmidt–Haensch polartronic HH8 polarimeter (Berlin, Germany) using a 1.0 cell. A high-performance liquid chromatography (HPLC) apparatus equipped with an LC-10ADVP pump, an SPD-M10AVP diode array detector and a DGU-14A degasser (Shimadzu, Kyoto, Japan) was used for both isolation and purity determination of the methylpluviatolide isomers, using an analytical Chiracel OJ (4.6 × 250 mm) column at a mobile phase flow rate (EtoH *n*-hexane: 65:35, v/v) of 0.9 mL/min and sample volume injection of 20 μL. The solvents used were of chromatographic grade.

4.2. Preparation of compounds

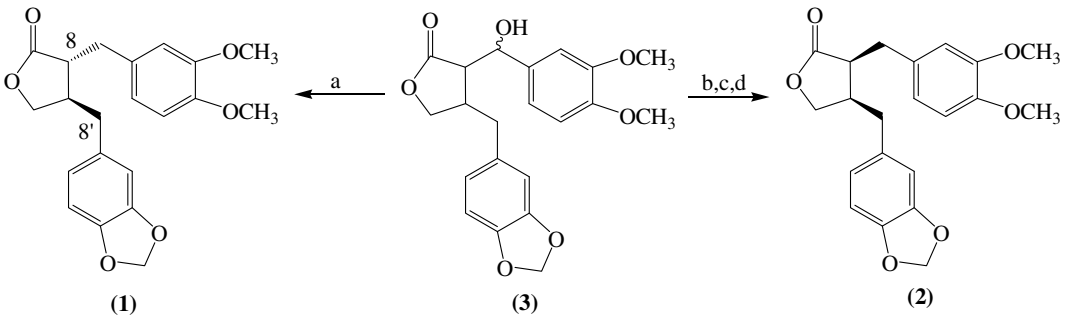
Cis and *trans*-methylpluviatolide were prepared from intermediate **3**, which was obtained by a procedure described by Landais et al. (1991) and Charlton and Chee (1997). Hydrogenolysis of **3** under the conditions described in Scheme 1 furnished the *trans*-steroisomer (**1**) in 80% yield. Acetylation of **3** followed by an elimination reaction with DBU produced a benzylidenebenzyl lignan, which furnished the *cis*-steroisomer (**2**) in 78% yield after hydrogenation (scheme 1).

Coupling constants of 15.2 Hz and 3.8 Hz between protons 8 and 8′ confirmed the *trans*- and *cis*-geometries for stereoisomers **1** and **2**, respectively.

The *trans*-methylpluviatolide racemic mixture was separated by chiral HPLC, and the fraction corresponding to each peak was collected and analyzed in the polarimeter for determination of the specific rotation of each enantiomer. The peak with a retention time of 36.20 min was identified as (+)-methylpluviatolide (**1**) $[\alpha]_D^{25}$ +20.8 (*c* 1.51, $CHCl_3$), and the peak with a retention time of 43.70 min was identified as (−)-methylpluviatolide (**1**) $[\alpha]_D^{25}$ -20.8 (*c* 1.51, $CHCl_3$).

4.3. Parasites and culture procedure

Trypomastigote forms of the Y strain of *T. cruzi* were used for the trypanocidal assay and were cultivated in the $LLMCK_2$ cell lineage (Norval, 1979). The cells were cultivated in RPMI-1640 medium (GIBCO), supplemented with 2 mM L-glutamine, 10 mM $NaHCO_3$, 100 U/mL penicillin, 100 μg/mL streptomycin, and 5% inactivated fetal calf serum. The culture was kept at 37 °C in an atmosphere of 5% CO_2 and 95% humidity. Blood samples containing trypomastigote forms were added to the culture cell at a ratio of 3:1, the supernatant was removed after 24 h, and the medium was replaced. After 7 days under the same culture condition, the supernatant was removed and centrifuged, furnishing free trypomastigote forms of the parasite for the bioassay. The free trypomastigote forms were transferred to a 96-well microtitre plate, each well was filled with 1×10^6 trypomastigote forms of the parasite, and each test compound was added to the wells. After 24-h incubation, activity was evaluated by using a colorimetric technique at 595 nm with the addition of a tetrazolium salt [MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], as described by Muelas-Serrano et al. (2000). Negative (solvent plus medium) and positive (gentian violet at 250 μg/



Reaction condition: (a) H_2, 4 atm, Pd/C, EtOH, $HClO_4$, 60 h, rt; (b) THF, Ac_2O, Et_3N, DMAP, 2h, rt; (c) DBU, CH_2Cl_2, 5h, rt; (d) H_2, 4 atm, Pd/C, EtOH, 4h, rt.

Scheme 1.

mL) controls were also run in parallel. All the assays were undertaken in triplicate.

4.4. Cytotoxicity assay

The cytotoxic action of the compound was evaluated by the MTT method as described by Sieuwerts et al. (1995). In a 96-well microtitre plate, LLC-MK2 cells (5×10^5 cells/well) were cultivated in RPMI-1640 medium supplemented as described above. The compound was evaluated at concentrations of 64, 256, 512, and 2560 μM for 24 h, at 37 °C, under a 5% CO_2 and 95% humidity atmosphere. After this period, the cells were incubated with MTT (5 mg/mL) for 240 min, and the reaction was interrupted by addition of acidified isopropanol. A microtitre plate reader (TECAN-SUNRISE) was used to obtain the results (570 nm). The negative (cells and solvent) and positive (cells treated with Triton X-100 20% solution) cytotoxicity controls were run in parallel. All assays were carried out in triplicate.

The formula % cytotoxicity = $[1 - (Y - M_d)/(N - M)] \times 100$ was used to calculate the cytotoxicity percentage, where Y is the mean optical density of wells with cells and different concentrations of compound; M_d is the mean optical density of wells with medium and different concentrations of compound; M is the mean optical density of wells with medium only; and N is the mean optical density of wells with cells and no compound (negative control). The level of 50% cellular cytotoxicity (CC_{50}) was obtained by a sigmoidal dose-response curve. Stock solutions were prepared by dissolving the compound in pure dimethylsulfoxide (DMSO), and aliquots of the stock solution were added to cultures to give a final concentration of DMSO lower than 0.2%.

4.5. Enzymatic inhibition studies

The enzymatic activities of the *T. cruzi* gGAPDH enzyme have been determined according to a previously reported procedure by Vieira et al. (2001), using the forward reaction. The percentage of remaining activity was calculated by comparison with an inhibitor-free control experiment.

4.6. Production of H_2O_2 and other peroxides

This activity was evaluated by quantification of the oxidation product of H_2DCFDA, a dichlorofluorescein compound (Carthcart et al., 1983). To this end, trypomastigote forms of *Y* and Bolivian strain of *T. cruzi* were cultured in the LLC-MK2 cell line (Hull et al., 1962) and suspended (1×10^6 parasites/ml) in a medium containing 125 mM sucrose, 65 mM KCl, and 10 mM Hepes-KOH, pH 7.2. Then, methypluviatolide (**1**) was added to the medium at a concentration of 32 μM, followed by addition of 5 μM H_2DCFDA, and the system was incubated at room temperature for 35 min. The parasites were then removed by centrifugation (8000*g*, 4 °C, 10 min), and the fluorescence of the supernatant was determined at 485 nm as described by Fang and Beattie (2003). *t*-Butyl hydroperoxide and a parasite-containing solution of 10% DMSO were used as positive and negative controls, respectively. The assays were performed in triplicate.

4.7. Nitric oxide measurement

The effects on the NO production by mouse peripheral macrophages were evaluated using the method previously reported by Giorgio et al. (1998). Briefly, peritoneal exudate cells were collected from the peritoneal cavities of male balb/c mice, previously injected intraperitoneally with 3% thioglycolate medium 96 h before, and washed with 6 mL of ice-cold phosphate-buffered saline (PBS). Cells were cultured in 96-well-flat-bottomed plates at 5×10^5 cells/well. The cultures were infected with *T. cruzi* at a 10:1 parasite:cell ratio. The methylpluviatolide was added to the medium at a concentration of 8, 32, and 128 μM. Cultures were incubated at 37 °C in 5% CO_2 for 24 h. Nitric oxide was measured in the supernatants by using the Griess reaction (Green et al., 1982). The Griess reagent was prepared by mixing equal volumes of 1% sulfanilamide in 5% H_2PO_4 and 0.1% naphthylethylene diamine dihydrochloride. Equal amounts of culture supernatant and Griess reagent were combined, and incubated for 10 min at room temperature. Absorbance measured at 540 nm was compared to a sodium nitrite standard curve and data were expressed in μM.

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