

## Mechanisms Involved in the Antinociceptive Effects of 7-Hydroxycoumarin

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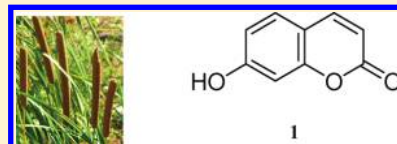
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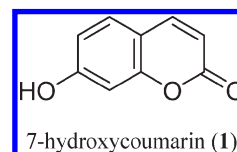
**ABSTRACT:** 7-Hydroxycoumarin (umbelliferone, **1**), the main metabolite of coumarin, has been reported to produce potent antinociceptive effects in animal models of pain. However, the biochemical events involved in antinociception mediated by **1** are currently not well understood. In the present study, the mechanisms by which **1** exerts its pharmacological actions were investigated. Acute pretreatment of mice with **1** produced a long-lasting antinociceptive effect against complete Freund's adjuvant (CFA)-induced hyperalgesia. The subchronic administration of **1** inhibited CFA-induced hyperalgesia and paw edema, while it did not cause any apparent toxicity. Another set of experiments showed that **1** inhibited carrageenan-induced mechanical hyperalgesia, but not mechanical hyperalgesia induced by prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), suggesting that it acts upstream of PGE<sub>2</sub>. Treatment with **1** was able to prevent the plantar tissue neutrophil influx induced by local inflammatory stimuli. In addition, **1** exhibited inhibitory effects on the release of hyperalgesic cytokines (TNF- $\alpha$  and IL-1 $\beta$ ) and the production of PGE<sub>2</sub>, a directly acting hyperalgesic mediator. The present results suggest that the antinociceptive effect of **1** is correlated with the inhibition of neutrophil migration, cytokine release, and PGE<sub>2</sub> production and are supportive of the further investigation of the therapeutic potential of **1** to control inflammatory pain.



Currently, many drugs prescribed worldwide are obtained directly or indirectly from plants, and there is a growing interest in the use of plants for the search for new therapeutic agents.<sup>1</sup> Coumarins constitute a large class of secondary metabolites widely distributed in the plant kingdom.<sup>2</sup> The main biotransformed product of coumarin (1,2-benzopyrone) in humans is 7-hydroxycoumarin (**1**), also known as umbelliferone, present in certain edible fruits and plants.<sup>3</sup> This compound has been found to exhibit a wide range of bioactivities, such as antioxidant,<sup>4,5</sup> antitumor,<sup>6,7</sup> antihyperglycemic,<sup>8</sup> antifungal,<sup>9</sup> antiasthmatic,<sup>10</sup> antipyretic, and anti-inflammatory effects.<sup>11,12</sup> In addition, we have recently reported that **1** given orally produced consistent dose-related and long-lasting antinociception in different models of pain, such as acetic acid-induced writhing, the formalin test, and complete Freund's adjuvant-induced hyperalgesia.<sup>13</sup> However, the mechanisms by which this molecule acts remain to be elucidated.

Pain is one of the classic signs of the inflammatory process. Inflammatory pain is induced by different chemical mediators released during this process and leads to nociceptive sensitization. The sensitization of primary nociceptive neurons is a common denominator of all kinds of inflammatory pain that result in a state of hyperalgesia (an increased response to a stimulus that is normally painful) and allodynia (pain due to a stimulus that does not normally provoke pain).<sup>14</sup> The present

study was focused on **1** as a putative modulator of different events and mediators involved with nociceptor sensitization and inflammatory pain, aiming to understand the mechanisms by which **1** induces antinociception in inflammatory conditions. In addition, the systemic toxicity associated with the daily oral administration of **1** was evaluated.

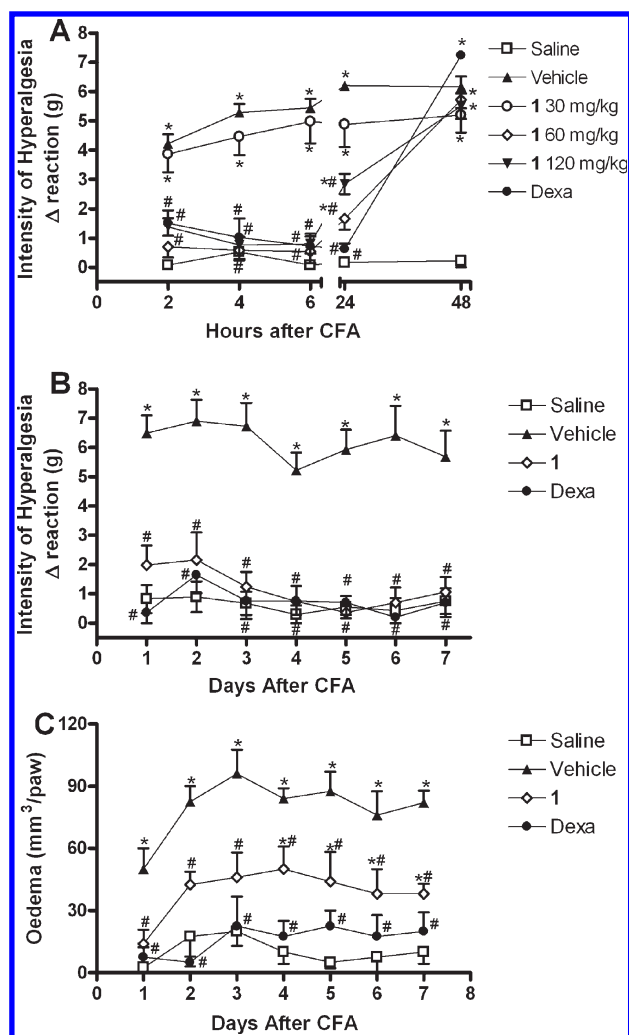


### RESULTS AND DISCUSSION

In the present study, the modulatory activity of **1** was examined on different events and mediators involved with inflammatory pain, aiming to understand the mechanisms by which **1** induces antinociception. Treatment of mice with **1** attenuated hyperalgesia and edema in response to inflammatory stimuli. Importantly, at a similar range of doses, **1** inhibited key events related to nociceptive sensitization and pain, namely, a

**Received:** September 3, 2010

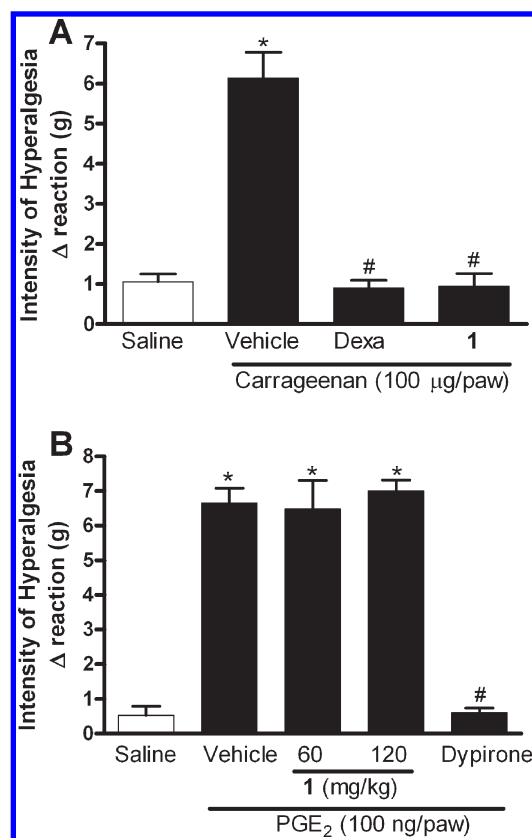
**Published:** March 18, 2011



**Figure 1.** Inhibition of hyperalgesia and edema induced using complete Freund's adjuvant (CFA) by 7-hydroxycoumarin (**1**) treatment. (A) Mice were treated with **1** (30–120 mg/kg), dexamethasone (dexa; 2 mg/kg), or vehicle (DMSO 2% in saline) 45 min before the intraplantar injection of CFA (10  $\mu$ L/paw). (B and C) Mice were treated with **1** (60 mg/kg), dexamethasone (2 mg/kg), or vehicle once a day for 7 days. The daily treatment started 45 min before CFA injection. Nociceptive threshold (B) and edema (C) were evaluated 3 h after the daily drug administration. The saline group received an intraplantar injection of saline (10  $\mu$ L/paw) instead of CFA. Data are reported as means  $\pm$  SEM;  $n = 6$  mice per group ( $*p < 0.05$  vs saline group;  $^{\#}p < 0.05$  vs vehicle-treated group; repeated measures of two-way ANOVA followed by the Bonferroni test).

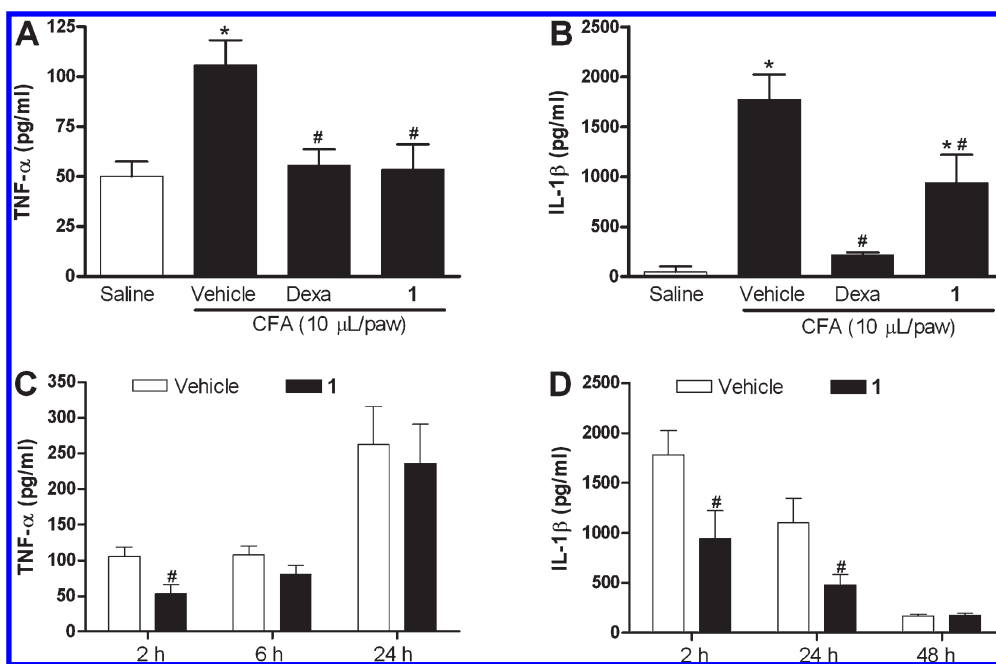
local increase of IL-1 $\beta$  and TNF $\alpha$  levels, production of PGE<sub>2</sub>, and neutrophil migration. These inhibitory effects may, at least in part, be responsible for the antinociceptive properties of **1**. Reinforcing the therapeutic potential of **1**, the daily administration of this compound did not induce detectable systemic toxicity or gastric injury.

In line with previous results,<sup>13</sup> oral administration of a single dose of **1** (30–120 mg/kg), 45 min before complete Freund's adjuvant (CFA), reduced significantly mechanical hyperalgesia 2, 4, 6, and 24 h after the intraplantar injection of CFA (10  $\mu$ L/paw) in a dose-dependent manner (Figure 1A). The results obtained with control groups support the effects of **1**, since the vehicle (DMSO 2% in saline) had no activity, and the standard drug



**Figure 2.** Effect of 7-hydroxycoumarin (**1**) on mechanical hyperalgesia induced by carrageenan (A) and PGE<sub>2</sub> (B). (A) Mice were treated orally with **1** (60 mg/kg), dexamethasone (dexa; 2 mg/kg), or vehicle (DMSO 2% in saline) 45 min before the intraplantar injection of carrageenan (100  $\mu$ g/paw). (B) Mice were orally treated with **1** (60 and 120 mg/kg), vehicle, or dypirone (160  $\mu$ g/paw) 45 min before the intraplantar injection of PGE<sub>2</sub> (100 ng/paw). Hyperalgesia was measured before and 3 h after injection of carrageenan or PGE<sub>2</sub> into the mice hind paw. The saline group received an intraplantar injection of saline (10  $\mu$ L/paw) instead of carrageenan or PGE<sub>2</sub>. Data are reported as means  $\pm$  SEM;  $n = 6$  mice per group ( $*p < 0.05$  vs saline group;  $^{\#}p < 0.05$  vs vehicle-treated group; one-way ANOVA followed by the Bonferroni test).

dexamethasone (2 mg/kg, sc) also inhibited CFA-induced hyperalgesia. It is important to mention that pretreatment with **1** did not alter the baseline nociceptive threshold of the test animals used (not shown in Figure 1A). To investigate the effects of long-term treatment, mice were treated once a day for seven days with **1** at 60 mg/kg, a dose that produced the maximal antinociception by acute administration. Mechanical hyperalgesia and paw edema induced by CFA were evaluated throughout the experimental period. Daily treatment with **1** markedly decreased the paw withdrawal response (Figure 1B). Importantly, the antinociceptive effect was maintained during the entire period of treatment, only returning to the control level two days after discontinuing daily treatment. Vehicle treatment did not affect the hyperalgesia observed, while the control drug dexamethasone inhibited CFA-induced hyperalgesia. The administration of **1** (60 mg/kg) produced a significant reduction in paw edema (Figure 1C). The edema was strongly inhibited by dexamethasone, the reference drug. Next, the antinociceptive effect of **1** was tested using different hyperalgesic stimuli, i.e., carrageenan and PGE<sub>2</sub>. Pretreatment with **1** (60 mg/kg) or the reference drug (dexamethasone; 0.5 mg/kg, sc), 45 min before

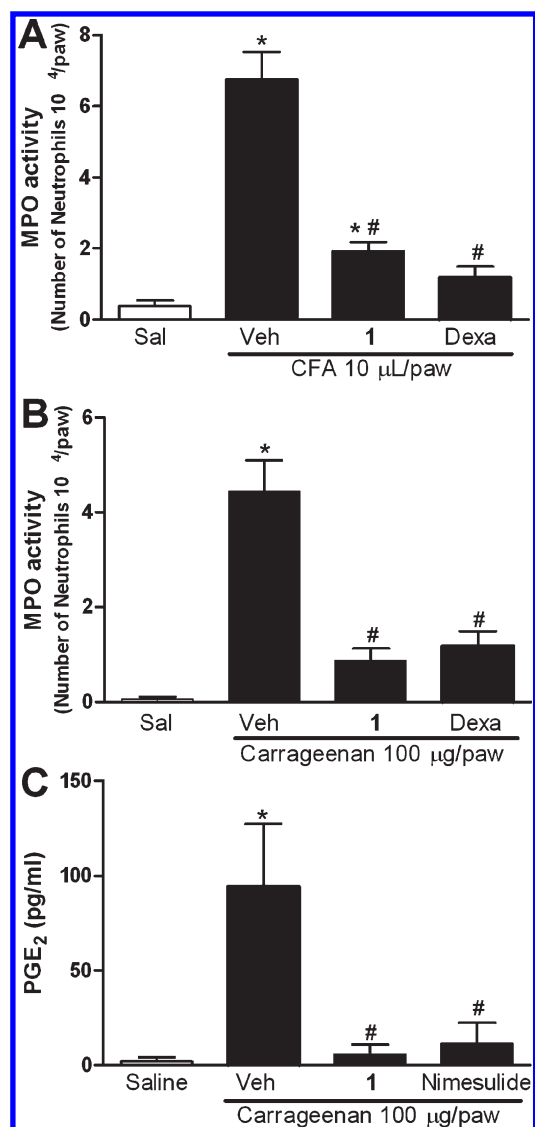


**Figure 3.** Effect of treatment with 7-hydroxycoumarin (**1**) on TNF- $\alpha$  (A and B) and IL-1 $\beta$  (C and D) levels. Mice were injected with **1** (60 mg/kg), vehicle (DMSO 2%; control), or dexamethasone (dexa; 2 mg/kg) by the oral route 45 min before the intraplantar injection of complete Freund's adjuvant (CFA; 10  $\mu$ L/paw). The saline group received an intraplantar injection of saline (10  $\mu$ L/paw) instead of CFA. The paw levels of TNF- $\alpha$  and IL-1 $\beta$  were measured by ELISA 2 h (A and B) or 2, 6, 24, and 48 h (C and D) after the CFA injection. Data are reported as means  $\pm$  SEM;  $n = 6$  mice per group (\* $p < 0.05$  vs saline group; # $p < 0.05$  vs vehicle-treated group; one-way ANOVA followed by the Bonferroni test).

the stimulus, inhibited carrageenan-induced hyperalgesia (Figure 2A). On the other hand, **1** (60 and 120 mg/kg, 45 min before stimuli) did not inhibit mechanical hyperalgesia induced by PGE<sub>2</sub> (Figure 2B). Treatment with dipyrone (160  $\mu$ g/paw) completely reversed the PGE<sub>2</sub>-induced hyperalgesia. Analgesic activity of **1** isolated from *Angelica pubescens* has been shown in a visceral pain model.<sup>11</sup> Recently, our group showed that **1** isolated from *Typha domingensis* produces potent antinociceptive effects in different models of pain.<sup>13</sup> The results presented here extend the literature data and clearly demonstrate that **1** administered by an oral route elicited a marked and long-lasting antinociception in inflammatory pain models. The acute administration of **1** inhibited hyperalgesia induced by CFA or carrageenan. On the other hand, high doses of **1** failed to reduce the hyperalgesia induced by PGE<sub>2</sub>, a direct-acting hyperalgesic mediator.<sup>15</sup> These results suggest that **1** produces antinociception by acting upstream of PGE<sub>2</sub>.

It is well recognized that the local injection of CFA produces inflammatory hyperalgesia initiated by peripheral nociceptor activation and local release of mediators, such as cytokines and prostanoids, which are involved in the sensitization of nociceptive pathways.<sup>16</sup> Previously, **1** was shown to suppress the secretion of proinflammatory cytokines induced by influenza virus infection in P388D1 cells and infected mice.<sup>17</sup> Since inflammatory cytokines, such as TNF- $\alpha$  and IL-1 $\beta$ , mediate inflammatory hyperalgesia, whether or not **1** is able to inhibit the release of these cytokines was evaluated. The intraplantar injection of CFA (10  $\mu$ L/paw) induced a significant increase in paw levels of TNF- $\alpha$  and IL-1 $\beta$  (Figure 3). Pretreatment with **1** (60 mg/kg) or dexamethasone (2 mg/kg, sc) inhibited the increase of TNF- $\alpha$  and IL-1 $\beta$  production induced by CFA (Figure 3A and B). The evaluation of the time profile of cytokine inhibition showed that the inhibitory effects of **1** persisted until 24 h for IL-1 $\beta$  (Figure 3D) and 2 h for TNF- $\alpha$  (Figure 3C). The results

obtained with control groups supported the effects of **1**, since the vehicle (DMSO 2% in saline) had no activity. A key role of IL-1 $\beta$  and TNF- $\alpha$  in inflammatory hyperalgesia has been demonstrated. It is known that a cascade of proinflammatory cytokines, which includes IL-1 $\beta$  and TNF- $\alpha$ , precedes the release of the final hyperalgesic mediators, i.e., prostaglandins and sympathetic amines.<sup>15,18</sup> Cytokine antagonists were able to reduce inflammatory hyperalgesia in mice, indicating that cytokine activation is an important step in the development of inflammatory pain.<sup>19</sup> IL-1 $\beta$  stimulates the expression of COX-2 and the subsequent release of prostaglandins.<sup>20,21</sup> Corroborating this idea, the local injection of IL-1 $\beta$  and TNF- $\alpha$  produces hyperalgesia dependent on the release of prostanoids.<sup>22</sup> Herein, we have shown that **1** reduced IL-1 $\beta$  and TNF- $\alpha$  levels. Since the inhibition of IL-1 $\beta$  and TNF- $\alpha$  release might lead to the inhibition of prostanoid production, it is possible that **1** acts by preventing the nociceptive sensitization through the inhibition of the production of mediators that sensitize the nociceptor. In fact, in the present study it is demonstrated that the carrageenan intraplantar injection (100  $\mu$ g/paw) induced a significant increase in PGE<sub>2</sub> levels in the mouse paw, and this event was fully prevented by pretreatment with **1** (60 mg/kg) or nimesulide (reference drug; 5 mg/kg) (Figure 4C). Since the sensitization of nociceptors is the common denominator of different types of pain, the reduction of IL-1 $\beta$ , TNF- $\alpha$ , and PGE<sub>2</sub> levels can be responsible for the antinociceptive effect of **1**. The fact that pretreatment with **1** failed to inhibit PGE<sub>2</sub>-induced hyperalgesia substantiates this idea. On the other hand, our previous study has demonstrated that both pre- and post-treatment with **1** produced potent antinociceptive effects.<sup>13</sup> The present data show that **1** is able to prevent nociceptive sensitization and also reduces inflammatory pain after its induction. On the basis of the analgesic action of NSAID and its COX inhibition effect, it is accepted that prostaglandins are important contributors to inflammatory pain.<sup>23</sup>



**Figure 4.** Treatment with 7-hydroxycoumarin (**1**) inhibits PGE<sub>2</sub> local production and paw neutrophil migration induced by inflammatory stimuli. (A and B) Mice were treated with **1** (60 mg/kg), vehicle (DMSO 2% in saline; control), or dexamethasone (dexa; 2 mg/kg) by oral route 45 min before the intraplantar injection of CFA (10 µL/paw) or carrageenan (100 µg/paw), respectively. Three and four hours after stimulus, subcutaneous plantar paw tissue were collected for MPO analysis (panels A and B, respectively). (C) Mice were orally treated with **1** (60 mg/kg), vehicle, or nimesulide (5 mg/kg) 45 min before the intraplantar injection of carrageenan (100 µg/paw). At 3 h after carrageenan injection, the subcutaneous plantar hind paw tissue was collected and the PGE<sub>2</sub> levels were determined by ELISA. Data are reported as means ± SEM; *n* = 6 mice per group. (\**p* < 0.05 vs saline group; #*p* < 0.05 vs vehicle-treated group; one-way ANOVA followed by the Bonferroni test).

Since treatment with **1** reduced the initiated inflammatory hyperalgesia but failed to inhibit PGE<sub>2</sub>-induced hyperalgesia, additional mechanisms may be involved in **1**-induced antinociception.

Experimental studies have demonstrated that neutrophil migration is important for the establishment of hyperalgesia induced by different inflammatory stimuli.<sup>24,25</sup> Thus, the potential usefulness of **1** was tested as a pharmacological tool to inhibit

**Table 1. Systemic Effects of Daily Treatment with 7-Hydroxycoumarin (**1**)<sup>a</sup>**

parameter	daily treatment (0.2 mL/po)	
	vehicle	<b>1</b> (60 mg/kg)
corporal mass variation (g)	0.58 ± 0.03	0.6 ± 0.04
liver (g/10 g body weight)	0.73 ± 0.07	0.72 ± 0.07
kidney (g/10 g body weight)	0.06 ± 0.0	0.07 ± 0.0
heart (g/10 g body weight)	0.05 ± 0.0	0.05 ± 0.0
total leukocytes × 10 <sup>3</sup> /mL	5.900 ± 0.8	6.725 ± 1.5
creatinine (mg/dL)	0.26 ± 0.05	0.27 ± 0.05
ALT (UI/L)	68.0 ± 12.8	51.0 ± 15.4
AST (UI/L)	145.7 ± 23.4	115.0 ± 22.8

<sup>a</sup> Values are expressed as means ± SEM for five mice per group using the Student's *t* test.

neutrophil migration induced by inflammation. Intraplantar injection of CFA (10 µL/paw) or carrageenan (100 µg/paw) induced a significant neutrophil migration to the paw skin tissue, 4 and 3 h after stimulus, respectively (Figure 4A and B). Administration of **1** (60 mg/kg) or dexamethasone (reference drug; 2 mg/kg) decreased the paw neutrophil migration. A recent paper has reported that emigrating neutrophils are not responsible for the production of hyperalgesic cytokines during inflammation, but may be involved in the production and/or release of PGE<sub>2</sub>.<sup>24</sup> In line with these data, the reduction of PGE<sub>2</sub> levels induced by **1**, and consequently its antinociceptive effect, could be attributed to both the reduction of cytokine levels and the lower influx of neutrophils.

The long-term administration of the available anti-inflammatory—analgesic drugs may lead to development of threatening gastrointestinal ulcers, bleeding, renal disorders, and immunosuppression.<sup>26–28</sup> Taking this possibility into consideration, the systemic effects of the oral daily administration of **1** were studied. Oral treatment with **1** (60 mg/kg) in a single dose scheme over seven consecutive days did not affect the animal corporal mass or the wet weight of animal organs such as the liver, kidney, and heart when compared to the control (vehicle-treated mice) animals (Table 1). All the organs evaluated exhibited a normal appearance and the absence of edema after daily treatment with **1**. Values obtained for creatinine, used as a parameter of renal function, did not differ from controls. Moreover, liver function, as evaluated via hepatic enzymes (alanine amine transferase and aspartate amine transferase), and the number of blood circulating leukocytes were not altered by treatment with **1**, in comparison to controls. Similarly, daily treatment with **1** was found safe from the viewpoint of gastric inflammatory events and ulcer induction at therapeutic dose levels. A stomach microscopical evaluation showed an intact mucosa with no inflammatory cell infiltrate, edema, erosion, or necrosis in mice treated daily with **1** (Table 2). In addition, **1** is a major metabolite of coumarin in humans,<sup>29</sup> and no adverse effects of coumarin have been reported in humans after continued treatment using doses of up to 7 g daily for 2 weeks.<sup>30,31</sup> The low toxicity in humans and the encouraging systemic effects reported here reinforce the potential of **1** for pharmacological development.

In contrast to nonsteroidal anti-inflammatory agents, inhibitors of cytokine production exhibit disease-modifying activities in animal models.<sup>32</sup> Thus, the down-regulation of cytokines by nontoxic agents is a therapeutic strategy for the control of



Table 2. Gastric Effects of Daily Treatment with 7-Hydroxycoumarin (1)<sup>a</sup>

treatment	inflammatory cells index	erosion index	edema index	necrosis index
vehicle (control group)	0.2 ± 0.2	0	0.6 ± 0.2	0
indomethacin (10 mg/kg)	4.0 ± 0.4 <sup>b</sup>	3.0 ± 0.6 <sup>b</sup>	2.6 ± 0.6 <sup>b</sup>	0
1 (60 mg/kg)	0.4 ± 0.2	0	0.7 ± 0.2	0

<sup>a</sup> Mice were orally injected daily with a single dose of **1** or vehicle for seven consecutive days. The positive control group received a single dose of indomethacin. Values are expressed as means ± SEM for five mice per group. Ten fields were analyzed per section. <sup>b</sup> Different from control group,  $p < 0.05$ , ANOVA with Tukey's posthoc.

inflammatory diseases. Overall, the present results suggest strongly that the antinociceptive effects of **1** are related to its ability to inhibit the production and/or release of cytokines and PGE<sub>2</sub> and to reduce neutrophil migration. These results highlight the need for further investigations of the possible use of **1** as a prototype compound for the development of new drugs to treat inflammatory pain.

## EXPERIMENTAL SECTION

**General Experimental Procedures.** Melting points were determined using a Geahaka model PF1500 version 1.0 apparatus and were not corrected. The NMR spectra were acquired on a Varian System 500 spectrometer, equipped with a XW4100 HP workstation. High-resolution mass spectra were recorded on a microTOF spectrometer (LC-IT-TOF model 225-07 100-34, Bruker) with positive ionization modes of the ESI source. Silica gel 60 (Merck) was used for column chromatography, and Si gel 60 PF<sub>254</sub> (Merck) was used for purification of compounds by preparative TLC. All solvents used were analytical grade (Merck).

Experiments were performed using male Swiss mice (22–28 g) raised and maintained at the Animal Facilities of the Gonçalo Moniz Research Center. Animals were housed in temperature-controlled rooms (22–25 °C), under a 12:12 h light–dark cycle, with access to water and food ad libitum until use. All behavioral tests were performed between 8:00 A.M. and 5:00 P.M., and animals were used only once. Animal care and handling procedures were in accordance with the guidelines of the International Association for Study of Pain (IASP) on the use of animals in pain research.<sup>33</sup> All efforts were made to minimize the number of animals used and their discomfort. Behavioral tests were done without knowing to which experimental group each mouse belonged.

**Plant Material.** The aerial parts of *Typha domingensis* Pers. (Typhaceae) were collected first in March 2002 in Bravo, Bahia, Brazil, and later in December 2005, near Santa Rita, Paraíba, Brazil. The plant was identified by Ana Maria Giulietti and Maria de Fátima Agra, and the voucher specimens (Giulietti et al. 2035 and Agra et Góis 5520) were deposited in the Herbaria HUEFS and JPB.

**Extraction and Isolation.** The aerial parts (2000 g) of *T. domingensis* were extracted with 95% ethanol at room temperature. Isolation of **1** from *T. domingensis* was performed at the Federal University of Paraíba, Brazil. The extract (150 g) was evaporated under a vacuum to yield a brown residue, which was suspended in methanol–H<sub>2</sub>O (3:7 v/v) and fractionated with chloroform and ethyl acetate. The chloroform phase (25 g) was subjected to column chromatography over silica gel, eluting with hexane, chloroform, and methanol mixtures in increasing order of polarity, yielding three coumarins and one cinnamic acid derivative. The ethyl acetate phase (25 g) was subjected to column chromatography over Sephadex LH-20 using methanol, yielding two flavonoids. The isolated compounds were identified as coumarin (0.0044%), scopoletin (0.0014%),<sup>34</sup> 7-hydroxycoumarin (**1**, 0.007%),<sup>35</sup> *p*-coumaric acid (0.0016%),<sup>36</sup> quercetin (0.0028%),<sup>37</sup> and isorhamnetin 3-*O*-glucoside (0.0039%),<sup>38</sup> based on NMR spectroscopic data and comparison with values reported in the literature.

*7-Hydroxycoumarin (1)*: white, amorphous solid; HRESIMS  $m/z$  163.0406 [M<sup>+</sup>], calcd for C<sub>9</sub>H<sub>7</sub>O<sub>3</sub>, 163.0390. This compound exhibited NMR data consistent with literature values.<sup>35</sup>

The percent purity of 7-hydroxycoumarin (**1**) used in the biological experiments as determined by HPLC was 98%.

**Inflammatory Pain Model.** Mice were slightly anesthetized with halothane and received 10 μL of CFA (1 mg/mL of heat-killed *Mycobacterium tuberculosis* in 85% paraffin oil and 15% mannide mono-leate; Sigma, St. Louis, MO) subcutaneously in the plantar region of the right hind paw, according to a method reported previously.<sup>39</sup> Mechanical hyperalgesia was measured by the electronic pressure-meter test described immediately below.

**Electronic Pressure-Meter Test.** Mechanical hyperalgesia was tested in mice according to the methodology previously reported.<sup>40</sup> In a quiet room, mice were placed in acrylic cages (12 × 10 × 17 cm) with wire grid floors, 15–30 min before the beginning of the test. This consisted in evoking a hind paw flexion reflex with a hand-held force transducer (Electronic von Frey; Insight, Ribeirão Preto, SP, Brazil) adapted to a 0.5 mm<sup>2</sup> polypropylene tip. A tilted mirror placed under the grid provided a clear view of the hind paws of the mice. The investigator was trained to apply the tip perpendicularly to the central area of the hind paw with a gradual increase in pressure. The end point was characterized by the removal of the paw followed by clear flinching movements. After the paw withdrawal, the intensity of the pressure was recorded automatically. The animals were tested before and after treatment. The results are expressed by a delta (Δ) withdrawal threshold (in g), which was calculated by subtracting the average of the last three measurements after treatment from the average of three measurements before treatment.

**Paw Edema Test.** The volume of the mouse paws was measured with a plethysmometer (Ugo Basile, Comerio, Italy) before (V<sub>0</sub>) the intraplantar injection of complete Freund's adjuvant (CFA) and 3 h after (V<sub>T</sub>), as described previously.<sup>41</sup> The amount of paw swelling was determined for each mouse, and the difference between V<sub>T</sub> and V<sub>0</sub> was taken as the edema value (mm<sup>3</sup>/paw).

**Cytokine Measurement.** Skin tissues were removed from the paws 2 h after the intraplantar injection of CFA (10 μL/paw) in mice terminally anesthetized from each experimental group. Tissue proteins were extracted from 100 mg tissue/mL PBS to which 0.4 M NaCl, 0.05% Tween 20, and protease inhibitors (0.1 mM PMSF, 0.1 mM benzethonium chloride, 10 mM EDTA, and 20 KI aprotinin A/100 mL) were added. The samples were centrifuged for 10 min at 3000g, and the supernatant was frozen at –70 °C for later quantification.<sup>42</sup> IL-1β and TNF-α levels were estimated using commercially available immunoassay ELISA kits for mice (R&D System, Minneapolis, MN), according to the manufacturer's instructions. The results are expressed as picograms of cytokine per milliliter of protein solution. As controls, the concentrations of these cytokines were determined in naive and vehicle-treated mice.

**Leukocyte Migration to the Paw Skin Tissue.** Neutrophil migration to the hind paw plantar tissue of mice was measured indirectly using a myeloperoxidase (MPO) kinetic-colorimetric assay, as previously described.<sup>25</sup> Three hours after the CFA (10 μL/paw) or

carrageenan (100  $\mu\text{g}/\text{paw}$ ) injection, animals were terminally anesthetized, and samples of subcutaneous plantar tissue were collected in 50 mM  $\text{K}_2\text{HPO}_4$  buffer (pH 6.0) containing 0.5% hexadecyl trimethylammonium bromide (HTAB) and kept at  $-80^\circ\text{C}$  until use. Samples were homogenized using a tissue homogenizer (Omni International, Kennesaw, GA) centrifuged at 16100g for 4 min, and the resulting supernatant was assayed for MPO activity determination at 450 nm using a spectrophotometer (SpectraMax 190, Molecular Devices, Sunnyvale, CA), with three readings in 1 min. The MPO activity of samples was compared to a standard curve of neutrophils. Briefly, 10  $\mu\text{L}$  of sample was mixed with 200  $\mu\text{L}$  of 50 mM phosphate buffer at pH 6.0, containing 0.167 mg/mL *o*-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. The results were presented as the MPO activity (number of neutrophils  $10^4/\text{paw}$ ).

**Determination of PGE<sub>2</sub> Levels.** The methodology used was similar to that previously described.<sup>43</sup> Male Swiss mice were treated with the vehicle (DMSO 2% in saline; control group), nimesulide (5 mg/kg/ip), or **1** (60 mg/kg/po), and after 45 min, they received an intraplantar injection of carrageenan (100  $\mu\text{g}$ ). Three hours later, mice were sacrificed using deep  $\text{CO}_2$  anesthesia, and paw skin tissue samples were collected and soaked in 2 mL of PBS containing heparin (5 IU/mL). Indomethacin (50  $\mu\text{g}/\text{mL}$ ) was added to prevent further production of PGE<sub>2</sub>. After homogenizing the samples, they were centrifuged at 1300g for 10 min at  $4^\circ\text{C}$ , and the supernatant was rapidly frozen and stored at  $-70^\circ\text{C}$  until analysis. For measurement of PGE<sub>2</sub> levels, a standard sandwich ELISA technique was used according to the recommendations of the manufacturer (R&D Systems, Minneapolis, MN).

**Evaluation of Systemic Effects of 1.** Corporal mass loss, organ weight alteration, gastric injury, a leukogram, and blood biochemical parameters (concentration of creatinine, alanine amino transferase/AST, and aspartate amino transferase/ALT) were evaluated after subchronic treatment with **1**. Animals were weighed and orally injected daily with a single dose of **1** (60 mg/kg) or the vehicle for seven consecutive days. At the end of treatment, blood samples were collected from the retro-orbital plexus of the mice under light anesthesia. Biochemical analyses were performed in serum samples obtained after centrifugation of total blood without anticoagulants, at 2500 rpm for 15 min. Standardized diagnostic kits by using a LABTEST spectrophotometer were used for determinations of the AST, ALT, and creatinine concentrations. For each leukogram determination, an aliquot of blood per animal was placed in EDTA and counts of leukocytes were carried out using a cell counter (Advia 60 Hematology System; Bayer, Tarrytown, NY). The liver, kidney, stomach, and heart of each animal were removed and weighed. The wet weight of each organ was expressed relative to 10 g of body weight and compared to the vehicle-injected group (control group).

In order to evaluate possible gastric effects from the daily administration of **1**, histopathological analysis of the stomach was performed. After removal, each stomach was opened through the greater curvature, washed with saline, fixed in 10% formalin solution, rinsed, dehydrated, and embedded in paraffin. Tissue blocks were sectioned at 3  $\mu\text{m}$  thickness (Cryostat CM 1800; Leica, Nussloch, Germany), stained with hematoxylin–eosin, and observed by light microscopy (40 $\times$ ) to examine morphological alterations, inflammatory cell infiltration, edema, epithelial cell loss, and necrosis. The histopathological alterations were assessed using the following scores: 0, absence; 1, discrete; 2, moderate; 3, moderate to intense; 4, intense.<sup>44</sup> For each stomach, 10 fields (40 $\times$ ) were analyzed per section, and the results were expressed as means  $\pm$  SEM. Morphological analyses were performed without knowing to which experimental group each mouse belonged. In order to illustrate severe inflammatory events and high ulcer incidence in mouse gastric tissue, an indomethacin-induced gastric ulcer model was used.<sup>45</sup>

**Test Compounds and Stimuli.** The test compounds used in this study were prostaglandin E<sub>2</sub>, complete Freund's adjuvant, dipyrone,

nimesulide, indomethacin, and dexamethasone from Sigma (St. Louis, MO) and carrageenan from FMC Corporation (Philadelphia, PA). Drugs were administered by oral (po), intraplantar (ipl), subcutaneous (sc), or intraperitoneal (ip) routes. The oral administration of 7-hydroxycoumarin was performed by gavage, and the control group received the vehicle only. Compound **1** was dissolved in 2% DMSO plus saline, and the remaining drugs were dissolved directly in saline.

**Statistical Analysis.** Data are presented as means  $\pm$  standard error of the mean (SEM) of measurements made on 5–7 animals in each group. Comparisons between three or more treatments were made using one-way ANOVA with Tukey's posthoc test or repeated measures of two-way ANOVA with Bonferroni's posthoc test, as appropriate. The results for ulcerogenic effects were analyzed as medians with their corresponding confidence limits (95%) and compared by the nonparametric Kruskal–Wallis test followed by the Dunn's posthoc test. All data were analyzed using Prism 4 Computer Software (GraphPad, San Diego, CA). Statistical differences were considered to be significant at  $p < 0.05$ .

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## ACKNOWLEDGMENT

This work was supported by grants from Fundação de Amparo à Pesquisa do Estado da Bahia (FAPESB), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Rede Nordeste de Biotecnologia (RENORBIO), and Conselho Nacional de Pesquisa (CNPq).

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