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Pimaradienoic Acid Inhibits Inflammatory Pain: Inhibition of NF-κB Activation and Cytokine Production and Activation of the NO−Cyclic GMP−Protein Kinase G−ATP-Sensitive Potassium Channel Signaling Pathway

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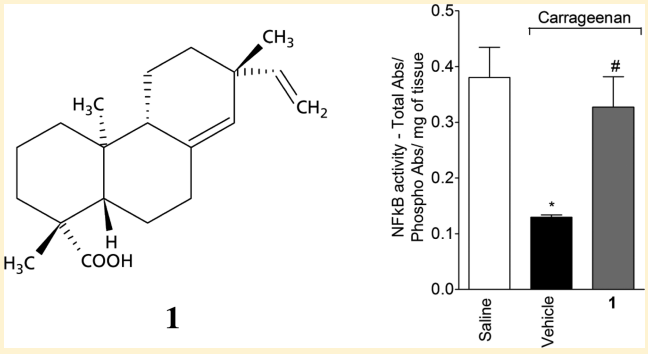
ABSTRACT: Pimaradienoic acid (**1**) is a pimarane diterpene (ent-pimara-8(14),15-dien-19-oic acid) extracted at high amounts from various plants including *Vigueira arenaria* Baker. Compound **1** inhibited carrageenan-induced paw edema and acetic acid-induced abdominal writhing, which are its only known anti-inflammatory activities. Therefore, it is important to further investigate the analgesic effects of **1**. Oral administration of **1** (1, 3, and 10 mg/kg) inhibited the acetic acid-induced writhing. This was also observed at 10 mg/kg via sc and ip routes. Both phases of the formalin- and complete Freund's adjuvant (CFA)-induced paw flinch and time spent licking the paw were inhibited by **1**. Compound **1** inhibited carrageenan-, CFA-, and PGE_2-induced mechanical hyperalgesia. Treatment with **1** inhibited carrageenan-induced production of TNF-α, IL-1β, IL-33, and IL-10 and nuclear factor κB activation. Pharmacological inhibitors also demonstrated that the analgesic effects of **1** depend on activation of the NO−cyclic GMP−protein kinase G−ATP-sensitive potassium channel signaling pathway. Compound **1** did not alter plasma levels of AST, ALT, or myeloperoxidase activity in the stomach. These results demonstrate that **1** causes analgesic effects associated with the inhibition of NF-κB activation, reduction of cytokine production, and activation of the NO−cyclic GMP−protein kinase G−ATP-sensitive potassium channel signaling pathway.



One of the most important symptoms of the inflammatory process is pain.[1] The carrageenan-induced inflammatory hyperalgesia (exacerbated pain due to stimulus that normally provokes pain) model shows that the release of prostaglandins (PGs) and sympathetic amines (e.g., dopamine, epinephrine) occurs subsequent to the release of a cascade of cytokines and other inflammatory mediators.[1−3] This cascade is initiated with the release of interleukin (IL)-33,[2] which induces the production of tumor necrosis factor α (TNF-α) and keratinocyte-derived chemokine 1 (CXCL1). TNF-α and CXCL1 induce the production of IL-1β, which in turn induces the production of PGs. CXCL1 is also responsible for stimulation of sympathetic amines release.[1−3] Both PGs and sympathetic amines induce a decrease in the nociceptor depolarization threshold, resulting in increases of neuronal membrane excitability, facilitating primary nociceptor activation and impulse transmission, which is observed as hyperalgesia.[1] On the other hand, the anti-inflammatory cytokine IL-10 limits inflammatory hyperalgesia by inhibiting the production of hyperalgesic cytokines such as TNF-α and IL-1β.[4] Thus, cytokines are important components of inflammatory pain, and modulating their production and action are conceivable approaches to control hyperalgesia.[5−8] Antinociception can also be achieved by activating the NO (nitric oxide)−cyclic-GMP (cyclic-guanosine monophosphate)−PKG (protein kinase G)−ATP-sensitive potassium channel signaling pathway. For instance, morphine,[9] some nonsteroidal anti-inflammatory drugs such as diclofenac[10] and dypirone,[11] synthetic molecules,[12,13] and natural products[14,15] induce analgesia by

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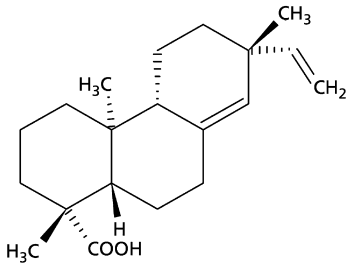
activation of the NO−cyclic-GMP−PKG−ATP-sensitive potassium channel signaling pathway.

Pimaradienoic acid (**1**) is a pimarane diterpene (ent-pimara-8(14),15-dien-19-oic acid) present in various plants and in higher concentration in *Vigueira arenaria* Baker (Asteraceae), a plant found in the herbaceous native savannas of Brazil.[16−18] This compound possesses a wide variety of activities, including antispasmodic and relaxant actions on vascular smooth muscle and inhibition of rat carotid contractions,[18,19] in part, by activating the NO−cGMP pathway and the release of arachidonic acid metabolites.[20] Furthermore, **1** also shows significant antimicrobial activity.[21,22] Regarding the anti-inflammatory effects of **1**, in vitro evidence shows that in RAW 264.7 macrophages it reduces NF-κB activation as well as LPS-induced production of NO and PGE_2, which correlated with inhibition of LPS-induced COX-2 and iNOS mRNA expression.[23] In vivo, there is only evidence that **1** significantly inhibits the carrageenan-induced inflammatory paw edema[24] and acetic acid-induced nociceptive writhing in mice.[25]

In view of the information presented above, we further addressed the analgesic effects of **1** in additional models and investigated whether its mechanism of action depends on modulation of cytokine production, NF-κB activation, and the NO−cGMP−PKG−ATP-sensitive potassium channel signaling pathway activation in mouse models of pain.



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RESULTS AND DISCUSSION

Pimaradienoic Acid (1) Inhibits the Writhing Response Induced by Acetic Acid. Mice received per oral (po) treatment with **1** (1−10 mg/kg, 2% DMSO diluted in saline) 30 min before intraperitoneal (ip) injection of acetic acid 0.8% diluted in saline. All doses of **1** inhibited the abdominal writhing (Figure 1A) with significant differences comparing the dose of 10 mg/kg and the other, lower doses. Thus, the dose of 10 mg/kg was selected for the next experiments on overt pain-like behavior models. Compound **1** administered ip (Figure 1B) and subcutaneous (sc) (Figure 1C) also inhibited the writhing response, demonstrating that it can be used by varied routes of administration depending on the therapeutic needs. The oral route was selected for subsequent experiments since it is less invasive, convenient, and accepted by patients and does not need a health care professional for administration.[26] The acetic acid model has nociceptive mechanisms that include the participation of inflammatory mediators, such as PGs that sensitize nociceptors and cytokines including TNF-α, IL-1β, and IL-33,[27,28] directly linked to inflammatory and nociceptive processes.[29] Furthermore, drugs that activate the NO−cyclic GMP−PKG−ATP-sensitive potassium channel signaling pathway reduce the

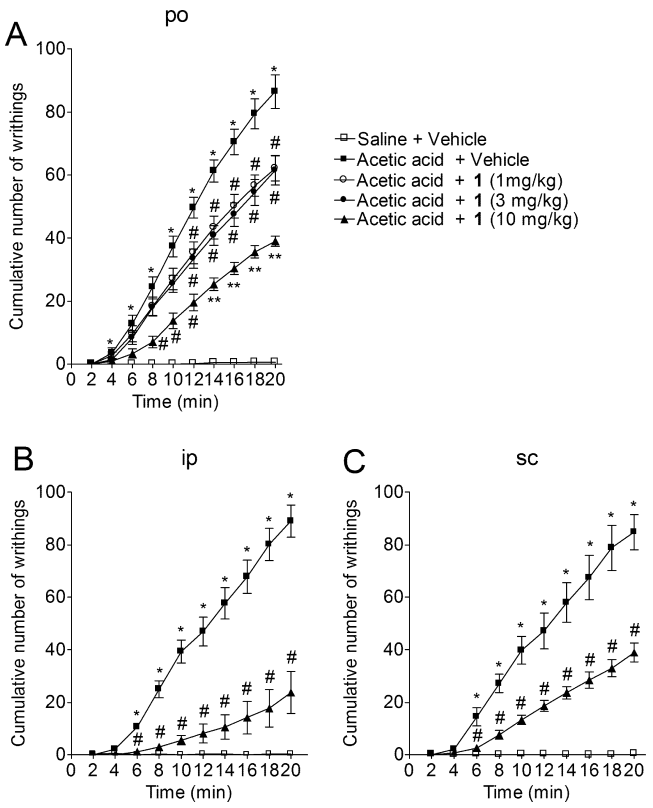

Figure 1. Pimaradienoic acid (**1**) inhibits acetic acid-induced writhing response. Mice were treated po (A), ip (B) or sc (C) with **1** (1−10 mg/kg) or vehicle (DMSO 2% diluted in saline) 30 min before acetic acid injection. The cumulative number of writhings was evaluated over 20 min and is presented at 2 min intervals. Results are provided as means ± SEM of six mice per group per experiment and are representative of two separate experiments [$^{*}p < 0.05$ compared to the saline group; $^{\#}p < 0.05$ compared to the vehicle group, $^{**}p < 0.05$ compared to the vehicle group and the doses of 1 and 3 mg/kg of **1** (one-way ANOVA followed by Tukey's test)].

number of abdominal contortions.[30] Therefore, it is possible that the antinociceptive activity of compound **1** may be related to modulation of these pathways. Previous studies reported that **1** administered at doses 30- to 50-fold higher than the dose used in the present study inhibited acetic acid-induced writhing.[25] Thus, analgesia can be achieved at much lower doses.

Pimaradienoic Acid (1) Inhibits Paw Flinch and Paw Licking Time Induced by Formalin and Complete Freund's Adjuvant (CFA). Mice were treated with **1** (10 mg/kg, po) 30 min before intraplantar (ipl) injection of formalin (1.5%, 25 μL). Compound **1** inhibited both phases of the formalin-induced paw flinches (Figure 2A) and licking (Figure 2B). In the first phase (0−5 min), direct activation of TRPA1 receptors present on primary nociceptive neurons[31] occurs along with the release of neurotransmitters such as bradykinin, serotonin, and histamine.[32] In the second phase (10−30 min), which is also called the inflammatory phase, there is a release of mediators such as TNF-α, IL-1β, IL-33, and PGs,[28,32,33] which are responsible for the activation and sensitization of primary sensory neurons. The activation of the NO−GMP signaling pathway inhibits formalin-induced overt pain-like behavior.[34] Therefore, **1** could potentially act on one or more of these targets to inhibit formalin-induced nociception. The CFA-induced inflammation depends on IL-

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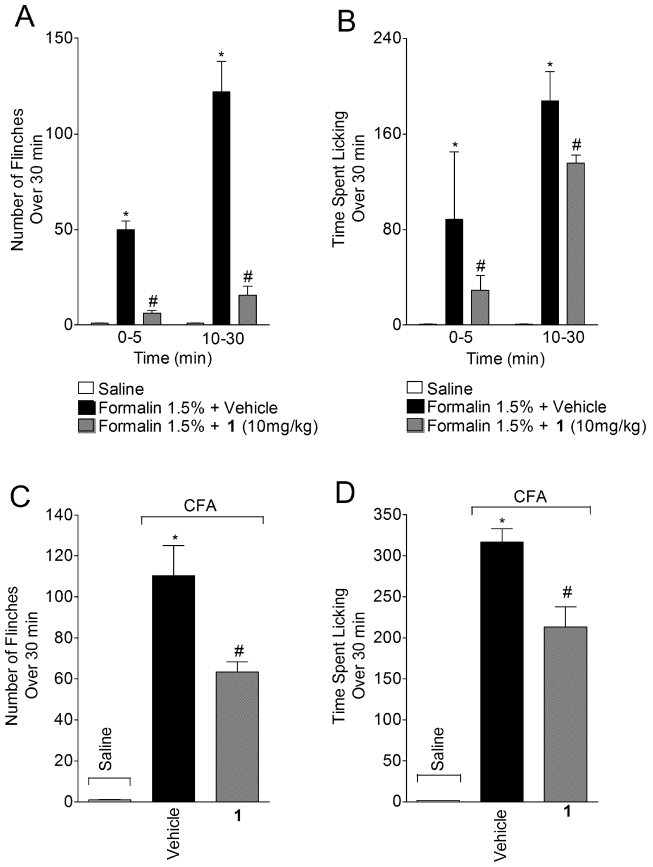

Figure 2. Pimaradienoic acid (**1**) inhibits paw flinch and time spent licking the paw induced by formalin and complete Freund's adjuvant (CFA). Mice were treated with **1** (10 mg/kg, po, 30 min) or vehicle before the injection of formalin (25 μL of 1.5% formalin in saline, panels A and B) or CFA (10 μL/paw, panels C and D). The total number of flinches (A and C) and time spent licking the paw (B and D) were evaluated for 30 min and expressed in intervals (0−5 and 10−30 min) for the formalin test and entire period (30 min) for the CFA test. Results are presented as means ± SEM of six mice per group per experiment and are representative of two separate experiments [$^{*}p < 0.05$ compared with the saline group, $^{\#}p < 0.05$ compared to the vehicle group (one-way ANOVA followed by Tukey's test)].

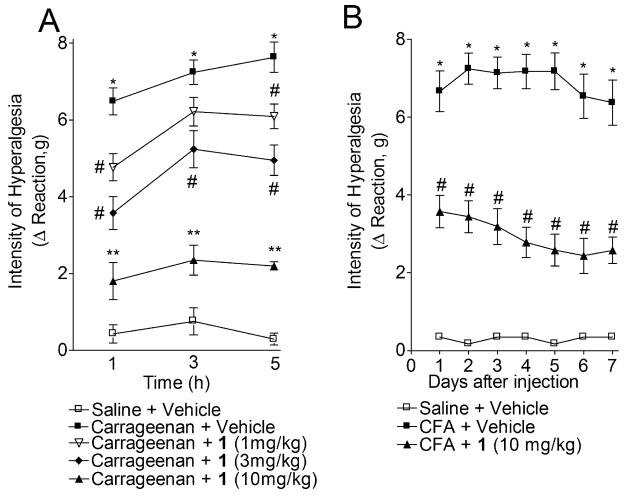

Figure 3. Pimaradienoic acid (**1**) inhibits carrageenan- and complete Freund's adjuvant (CFA)-induced mechanical hyperalgesia. Mice were treated with **1** (1−10 mg/kg, po, 30 min) or vehicle before carrageenan (300 μg/paw) or CFA (10 μL/paw) injection. The intensity of mechanical hyperalgesia was measured 1−5 h after carrageenan injection (A) or 1−8 days after CFA injection (B). Results are presented as means ± SEM of six mice per group per experiment and are representative of two separate experiments [$^{*}p < 0.05$ compared to the saline group; $^{\#}p < 0.05$ compared to inflammatory stimulus group; $^{**}p < 0.05$ compared to the carrageenan group and with the doses of 1 and 3 mg/kg of **1** groups (one-way ANOVA followed by Tukey's test)].

1β, TNF-α, and PGE_2 production, which induce nociceptor sensitization.[35,36] Pretreatment with **1** (10 mg/kg, po, 30 min) reduced paw flinch (Figure 2C) and licking (Figure 2D) behaviors induced by CFA (10 μL ipl), suggesting that the analgesic effect of **1** may be dependent on targeting cytokine production.

Pimaradienoic Acid (1) Inhibits Mechanical Hyperalgesia Induced by Carrageenan and CFA. Mice were treated with **1** (1−10 mg/kg, po) or vehicle 30 min before carrageenan (300 μg/paw) administration, and mechanical hyperalgesia was evaluated at 1−5 h after stimulus injection. Treatment with **1** (1−10 mg/kg) inhibited in a dose-dependent manner mechanical hyperalgesia (Figure 3A) induced by carrageenan. The dose of 1 mg/kg inhibited carrageenan-induced mechanical hyperalgesia at 1 and 5 h (Figure 3A). The analgesic effect of the doses of 3 and 10 mg/kg in carrageenan-induced mechanical hyperalgesia was significant at all evaluated time points. Importantly, the analgesic effect of 10 mg/kg was significantly better than the lower doses of **1** (Figure 3A). Therefore, the dose of 10 mg/kg of **1** was selected for subsequent experiments. The carrageenan-induced hyperalgesia model depends on the production of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-33) and PGE_2.[1−4] Previous in vitro studies have demonstrated that **1** inhibits LPS-induced production of IL-6 and cyclooxigenase-2 expression,[23] which is consistent with our data.

To further address the possible therapeutic usefulness of **1**, mice received ipl injection of CFA (10 μL/paw) and were treated with **1** (10 mg/kg, po) daily during 7 days, starting 24 h after CFA stimulus injection. Mechanical hyperalgesia was evaluated daily 3 h after treatment (Figure 3B). There was significant inhibition of mechanical hyperalgesia (Figure 3B) at all evaluated time points during treatment with **1**, demonstrating the antinociceptive effect of the compound in another inflammatory pain model and its potential applicability in pain and inflammation. Similarly to the overt pain-like behavior induced by CFA, the mechanical hyperalgesia also depends on cytokines and can be inhibited by compounds or drugs that modulate cytokine production.[35,36]

Pimaradienoic Acid (1) Inhibits Carrageenan-Induced Cytokine Production. Mice were treated with **1** (10 mg/kg, po) 30 min before carrageenan ipl injection (300 μg/paw), and after 3 h samples of cutaneous plantar tissue were collected for cytokine level measurement (Figure 4). Treatment with **1** significantly reduced carrageenan-induced production of TNF-α (Figure 4A), IL-1β (Figure 4B), IL-33 (Figure 4C), and IL-10 (Figure 4D). Supporting these data, **1** significantly inhibited the production of the cytokine IL-6 in LPS-stimulated RAW 264.7 macrophages.[30] Importantly, TNF-α, IL-1β, IL-6, and IL-33 contribute to mechanical hyperalgesia and overt pain-like behaviors (abdominal writhing, paw flinch, and licking), and inhibition of their production is an important antinociceptive mechanism.[1−5,8,35,36] IL-10 is an anti-inflammatory cytokine that is co-released with pro-inflammatory cytokines and has a

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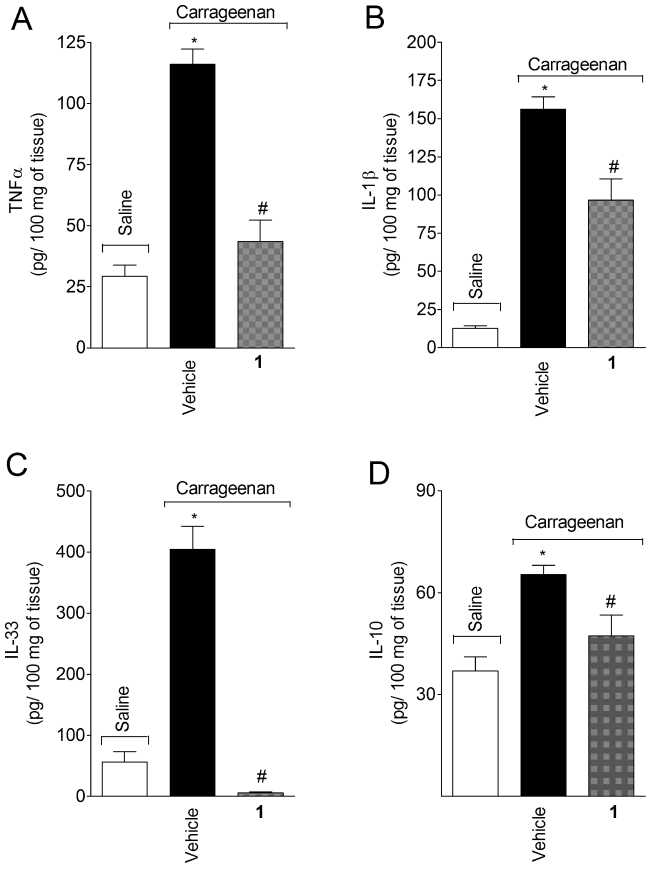

Figure 4. Pimaradienoic acid (**1**) inhibited carrageenan-induced TNF-α, IL-1β, IL-33, and IL-10 production. Mice were treated with **1** (10 mg/kg, po, 30 min) or vehicle before the ipl injection of carrageenan. Three hours after carrageenan injection, mice were euthanized and cutaneous plantar skin samples were collected for measurement of TNFα (A), IL-1β (B), IL-33 (C), and IL-10 (D) levels. Results are presented as means ± SEM of six mice per group per experiment and are representative of two separate experiments [$^{*}p < 0.05$ compared with the saline group, and $^{\#}p < 0.05$ compared to the vehicle group (one-way ANOVA followed by Tukey's test)].

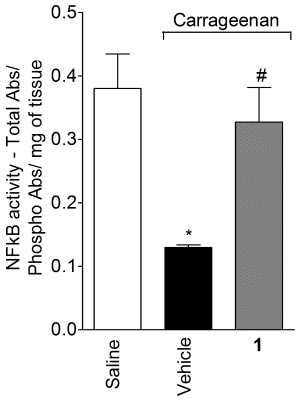

Figure 5. Pimaradienoic acid (**1**) inhibits carrageenan-induced NF-κB activation. Mice were treated with **1** (10 mg/kg, po, 30 min) or vehicle before the ipl injection of carrageenan. Three hours after carrageenan injection, mice were euthanized and paw skin samples were collected for the determination of NF-κB levels. Results are presented as means ± SEM of five mice per group per experiment and are representative of two separate experiments [$^{*}p < 0.05$ compared with the saline group, and $^{\#}p < 0.05$ compared to the vehicle group (one-way ANOVA followed by Tukey's test)].

role as an endogenous down-regulator of inflammatory pain since targeting IL-10 increases inflammation and pain and administration of IL-10 reduces nociceptive behaviors.[1,4] The present data are inconsistent with the hypothesis that analgesic effects of **1** depend on an increase of IL-10 levels. It is possible that the reduction of pro-inflammatory cytokine production resulted in reduced IL-10 levels considering that its role as an endogenous down-regulator was not necessary to control pro-inflammatory cytokine nociceptive effects.[1,4] To our knowledge, this is the first study to demonstrate that **1** inhibits TNFα, IL-1β, IL-33, and IL-10 production.

Pimaradienoic Acid (1) Inhibits Carrageenan-Induced Nuclear Factor κB (NF-κB) Activation. Mice were treated with **1** (10 mg/kg, po) 30 min before ipl injection of carrageenan (300 μg/paw). Carrageenan induced the activation of NF-κB at 3 h, as observed by the decreased ratio of total NF-κB/phosphorylated NF-κB, and treatment with **1** inhibited the carrageenan-induced NF-κB activation (Figure 5). NF-κB is a major transcription factor involved in the production of pro-inflammatory cytokines, COX-2 expression-dependent PGE_2 production, and iNOS (inducible nitric oxide synthase)-derived NO.[37−39] Targeting NF-κB is an effective pharmacological approach to inhibit pain.[40] Cytokine-induced receptor signaling activates NF-κB, resulting in up-regulation of cytokine production.[37−39] Therefore, the reduced cytokine production observed (results of Figure 4) is tightly related to reduced NF-κB activation. Thus, compound **1** could be acting by reducing cytokine activity in addition to cytokine production. Consistent with this last hypothesis, **1** inhibited TNFα-induced matrix metalloprotease-9 secretion and human aortic smooth muscle cell migration in vitro by targeting the transcription factor NF-κB and activating protein-1 (AP-1).[41] The present in vivo results demonstrating that **1** inhibits carrageenan-induced NF-κB activation are consistent with reports of its inhibitory activity over LPS-induced NF-κB activation in RAW267.4 macrophages[23] and TNFα-induced NF-κB activation in human aortic smooth muscle cells.[41]

Pimaradienoic Acid (1) Activates the NO−Cyclic GMP−PKG−ATP-Sensitive Potassium Channel Signaling Pathway. Mice were treated with **1** (10 mg/kg, po) or vehicle 30 min before ipl injection of PGE_2 (100 ng/paw) (Figure 6). PGE_2 induced significant mechanical hyperalgesia compared to the saline group, and treatment with **1** inhibited PGE_2-induced mechanical hyperalgesia at 3 h (Figure 6). Compound **1** inhibited LPS-induced PGE_2 production by cultured macrophages.[23] The present data add to this finding by demonstrating that **1** also inhibits the hyperalgesic action of PGE_2. Because PGE_2 can directly induce nociceptor sensitization by activating its receptors in neurons, the analgesic effect of **1** might also involve activation of neuronal analgesic mechanisms in addition to peripheral mechanisms that contribute to nociceptor sensitization.[1,37]

Previous reports have shown that the relaxant action of **1** on vascular smooth muscle is partially dependent on the release of NO from endothelial cells, resulting in activation of guanylyl cyclase.[42] NO donors[43−45] and analgesics that release NO such as opioids[9] and dipyrone[11] inhibit inflammatory hyperalgesia including that induced by PGE_2 by activating the cyclic GMP−PKG−ATP-sensitive potassium channel signaling pathway. This is in line with the reduced potassium channel activity in many types of inflammatory pain.[46] Therefore, the next step was designed to investigate the possible activation of the NO−

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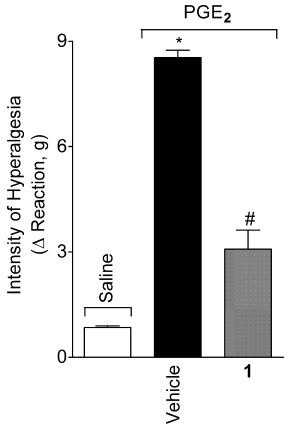

Figure 6. Pimaradienoic acid (**1**) inhibits PGE_2-induced mechanical hyperalgesia. Mice were treated with **1** (10 mg/kg, po) 30 min before PGE_2 (100 ng/paw) injection, and mechanical hyperalgesia was evaluated after 3 h. Results are presented as means ± SEM of six mice per group per experiment and are representative of two separate experiments [$^{*}p < 0.05$ compared with the saline group, $^{\#}p < 0.05$ compared to PGE_2 + vehicle group (one-way ANOVA followed by Tukey's test)].

GMP−PKG−ATP-sensitive potassium channel signaling pathway by **1**.

Mice were treated with L-NAME (Figure 7A; L-nitroarginine methyl ester, nonselective NOS inhibitor, 90 mg/kg, ip, diluted in saline, 60 min), ODQ (Figure 7B; a soluble guanylyl cyclase inhibitor, 0.3 mg/kg, diluted in 2% DMSO in saline, 30 min), KT5823 (Figure 7C; an inhibitor of PKG, 0.5 μg/animal, diluted in 2% DMSO in saline, 5 min), or glybenclamide (Figure 7D; an inhibitor of ATP-sensitive potassium channels, 0.3 mg/kg, diluted in 20% Tween 80 in saline, 45 min) before **1** (10 mg/kg, po) treatment. After an additional 30 min mice received an ipl injection of carrageenan (300 μg/paw), and mechanical hyperalgesia was evaluated after 1, 3, and 5 h. The inhibition of carrageenan-induced hyperalgesia by **1** was prevented by L-NAME, ODQ, KT5823, and glybenclamide treatments at 1, 3, and 5 h (Figure 7). Control treatments with L-NAME, ODQ, KT5823, or glybenclamide in mice that received saline ipl administration did not alter the nociceptive threshold of mice (data not shown). Therefore, the antinociceptive effect of **1** depends on the induction of NO production and consequent activation of the cyclic GMP−PKG−ATP-sensitive potassium channel signaling pathway, which diminishes the nociceptive neurotransmission, resulting in diminished inflammatory mechanical hyperalgesia.

The LPS-induced iNOS expression and NO production was inhibited by **1**,[23] indicating it inhibits excessive NO production in inflammation, which corroborates the inhibition of NFκB activation that would result in iNOS expression (Figure 5).[23] On the other hand, it is likely that **1** induces NO production at low levels by the constitutive endothelial (e) and neuronal (n) NOS isoforms, which would have an important signaling role for cellular communication. For instance, **1** induced the activation of the analgesic NO−GMP−PKG−ATP-sensitive potassium channel signaling pathway and NO-derived endothelial cell-dependent vasorelaxation.[42] Thus, **1** seems to have a dual role in NO production.

Pimaradienoic Acid (1) Does not Induce Hepatic Toxicity or Gastric Inflammation. In order to assess if **1** induces hepatic toxicity or gastric inflammation, plasma levels

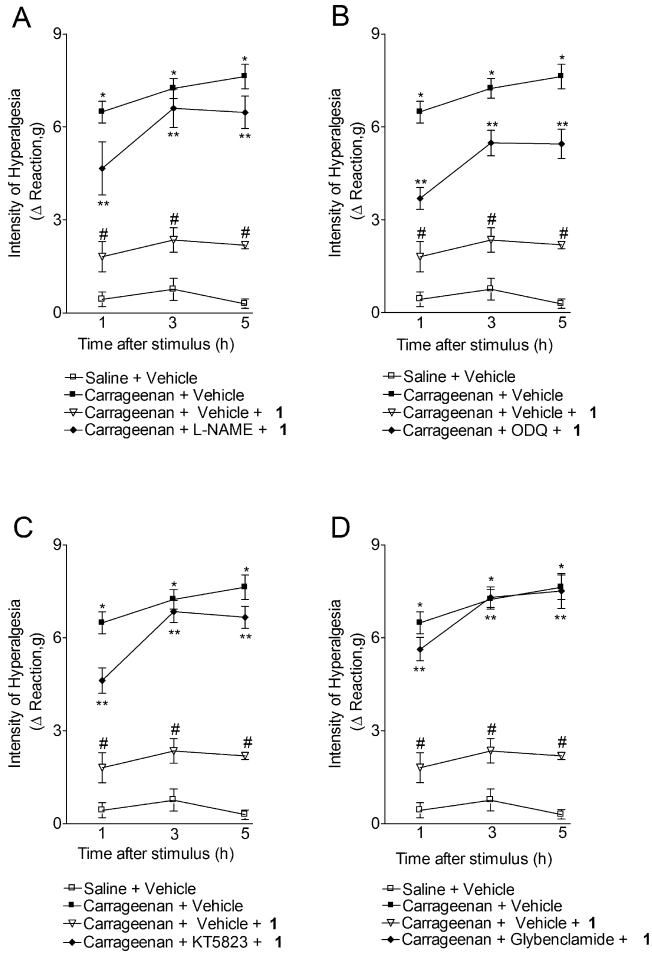

Figure 7. Pimaradienoic acid (**1**) inhibits inflammatory pain by activating the NO−cyclic GMP−protein kinase G−ATP-sensitive potassium channel signaling pathway. Mice were treated with L-NAME (A, NOS inhibitor; 90 mg/kg, ip, diluted in saline, 60 min), ODQ (B, soluble cGMP inhibitor; 0.3 mg/kg, ip, diluted in 2% DMSO in saline, 30 min), KT5823 (C, PKG inhibitor; 0.5 μg/mice, ip, diluted in 2% DMSO in saline, 5 min), or glybenclamide (D, ATP-sensitive potassium channel blocker; 0.3 mg/kg, ip, diluted in 5% Tween 80 in saline, 45 min) before treatment with **1** (10 mg/kg, po) and after an additional 30 min received carrageenan stimulus (300 μg/paw). Mechanical hyperalgesia was evaluated 1−5 h after carrageenan injection (A−D). Results are presented as means ± SEM of six mice per group per experiment and are representative of two separate experiments [$^{*}p < 0.05$ compared with the saline group, $^{\#}p < 0.05$ compared to carrageenan + **1** vehicle control group, $^{**}p < 0.05$ comparing the group treated with **1** and the groups cotreated with **1** and L-NAME (A), with **1** and ODQ (B), with **1** and KT5823 (C), and with glybenclamide (D) (one-way ANOVA followed by Tukey's test)].

of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and gastric mucosa myeloperoxidase (MPO) activity were determined using the same treatment protocol. Mice were treated daily (during 7 days) with **1** (10 mg/kg, po), vehicle (DMSO 2% diluted in saline or Tris/HCl buffer, pH 8.0, with the data for the vehicles pooled since there is no difference between the vehicles), or indomethacin (2.5 mg/kg).[26] No significant difference was observed in plasma levels of AST (Figure 8A) or ALT (Figure 8B) between mice treated with **1** or vehicle. On the other hand mice treated with the positive control indomethacin (a nonselective cyclooxygenase inhibitor) presented significant increases in AST

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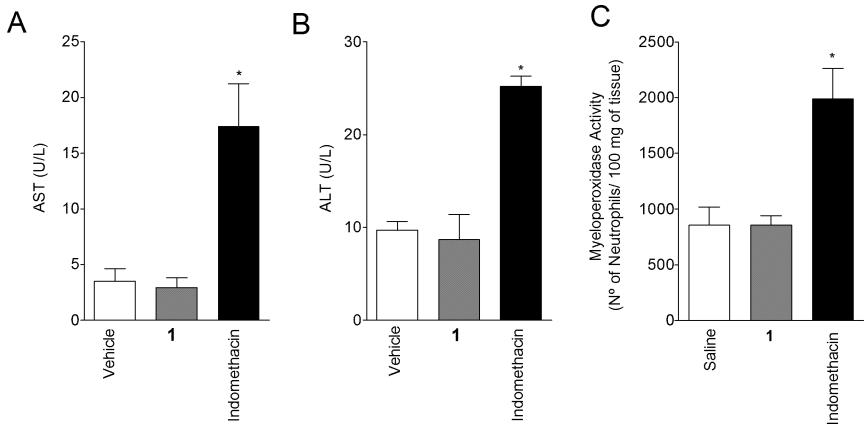

Figure 8. Pimaradienoic acid (**1**) does not increase plasma levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), or myeloperoxidase (MPO) activity in the stomach. Mice were treated po daily for 7 days with **1** (10 mg/kg), vehicle (DMSO 2% diluted in saline or Tris/HCl buffer, pH 8.0), or indomethacin (2.5 mg/kg). After 7 days of treatment, mice were terminally anesthetized, and blood and stomach samples were collected for AST (A) and ALT (B) plasma level or gastric MPO activity (C) determination, respectively. Results are presented as means ± SEM of eight mice per group per experiment and are representative of two separate experiments [$*p < 0.05$ compared with the saline group or **1** groups (one-way ANOVA followed by Tukey's test)].

and ALT plasma levels when compared to those treated with saline or **1**. AST and ALT are enzymatic markers of liver injury widely used to assess hepatotoxicity of drugs;[15,26] thus, these results indicate that seven daily treatments with **1** at 10 mg/kg does not induce hepatic toxicity.

Increase of MPO activity is a hallmark of nonselective COX inhibitors-induced gastric inflammatory lesions.[47] Accordingly, indomethacin induced a significant increase in MPO activity in stomach tissue compared to the vehicle group (Figure 8C). No statistical difference between groups treated with vehicle or **1** was observed (Figure 8). Therefore, **1** does not induce stomach inflammation, as assessed over a seven-day period of treatment. In agreement, **1** weakly inhibited LPS-induced PGE_2 production by RAW264.7 macrophages,[23] suggesting that inhibition of cyclooxigenase-1 activity (isoform whose inhibition is related to gastric ulcer formation)[47] is not a major mechanism of action of this diterpene.

Further corroborating the safety of **1** at doses with analgesic activity (10 mg/kg), it did not induce micronuclei formation or DNA damage up to 40 mg/kg in Swiss mice. Compound **1** also reduced the frequency of doxorubicin- and methylmethanesulfonate-induced micronuclei and extent of DNA damage in vivo and in vitro.[48] These data demonstrate that compound **1** does not present genotoxic effects at analgesic dose and rather possesses antigenotoxic activities in mammalian cells.

Pimaradienoic Acid (1) Showed No Muscle-Relaxing or Sedative Effects. Oral administration of **1** at 10 mg/kg did not alter the motor response of test animals 1.5, 3.5, and 5.5 h after treatment ($n = 6$). These time points were based on the 30 min of pretreament plus 1, 3, and 5 h until mechanical hyperalgesia measurement in the carrageenan model. The vehicle control response in the rota-rod test was 180 s versus 180 s of **1**-treated animals (data not shown). These results support the notion that **1** diminishes the nociceptive threshold induced by inflammation and not that mice are incapable of responding because of muscle-relaxing or sedative effects.[49]

Pimaradienoic Acid (1) Did Not Exhibit Any Effect in the Hot Plate Test in Naive Mice. Mice were treated with **1** (10 mg/kg, po route) or morphine hydrochloride (8 mg/kg, ip route), and the thermal nociception was evaluated ($n = 6$). Morphine hydrochloride treatment increased the thermal threshold of mice (data not shown) as expected because of its central analgesic effects. On the other hand, **1** did not alter the thermal threshold of mice (data not shown). This result further supports a peripheral neuronal effect of **1** upon inflammatory pain, since the hot plate test is considered to be modulated by supraspinal mechanisms.[50−52]

In conclusion, the present study demonstrates that **1** exerted analgesic effects in varied models in vivo. The mechanisms of action of **1** may involve the inhibition of cytokine production, inhibition of NF-κB activation, and activation of the NO−cyclic GMP−protein kinase G−ATP-sensitive potassium channel signaling pathway. Therefore, these data suggest that **1** may have therapeutic potential for the treatment of inflammatory pain conditions.

■ EXPERIMENTAL SECTION

General Experimental Procedures. The optical rotation of **1** was measured in $CHCl_3$ using a PerkinElmer 241 polarimeter. Nuclear magnetic resonance (NMR) spectra were run on a Bruker DPX 400 spectrometer (400 MHz for ^1H and 100 MHz for ^{13}C). Samples were dissolved in $CDCl_3$, and the spectra were calibrated with the solvent signals at δ 7.26 (^1H) and 77.0 (^{13}C). Mass spectrometric analysis was performed at low resolution on a Micromass Quattro-LC instrument (Manchester, UK) provided with an ESI ion source and a triple quadrupole mass analyzer. Solutions were dissolved in MeOH−H_2O 8:2 (v/v) and infused into the ESI source at a flow rate of 5 μL/min, using a Harvard apparatus model 1746 (Holliston, MA, USA) syringe pump. Vacuum-liquid chromatography (VLC) was carried out using silica gel 60H (Merck, art. 7736) in glass columns with 5−10 cm i.d.[29] High-performance liquid chromatography (HPLC) analysis was accomplished using a Shimadzu CBM-20A liquid chromatography controller, operating with LC solution software, equipped with a Shimadzu UV-DAD detector SPD-M20A and a Shimadzu ODS column (4.6 × 250 mm, 5 μm, 100 Å).[17,18]

During the experiments, mice received per oral, subcutaneous, or intraperitoneal treatment with pimaradienoic acid (**1**; 1, 3, and 10 mg/kg) or vehicle (saline) 30 min before or 24 h after inflammatory stimulus, as indicated. The doses of inflammatory stimuli were determined previously in our laboratory in pilot studies and are based on previous work.[2,3,9,14,15] The writhing response was evaluated during 20 min after ip injection of acetic acid (0.8%). The paw flinching and licking nociceptive responses were quantified for 30 min after formalin 1.5% (25 μL/paw) or CFA (10 μL/paw) injection. Mechanical hyperalgesia was evaluated 1−5 h after carrageenan (300 μg/paw) or

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1−7 days after CFA (10 μL/paw) stimulus. All inflammatory stimuli induced only ipsilateral (in the paw in which the stimulus was injected) mechanical hiperalgesia. TNFα, IL-1β, IL-33, and IL-10 levels were evaluated 3 h after carrageenan (300 μg/paw) injection. The plasma levels of AST and ALT and MPO activity in stomach samples were determined in mice treated po daily with vehicle, **1** (10 mg/kg), or indomethacin (2.5 mg/kg) for 7 days. The inflammatory pain models and time points of sample collection were determined previously in our laboratory in pilot studies and are based on previous work.[2,3,12]

Plant Material. *Viguiera arenaria* Baker (Asteraceae) was collected by F. B. Da Costa from the vicinity of the Washington Luís highway (km 223, 22°10 S, 47°59 W, SP, Brazil, in March 1999). The plant material was identified by J. N. Nakajima (Universidade Federal de Uberlândia, MG, Brazil) and E. E. Schilling (University of Tennessee, TN, USA). A voucher specimen (FBC 60) was deposited under code SPFR 4006 in the herbarium of the Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, SP, Brazil.[17,18]

Extraction and Isolation. Air-dried tuberous roots (980 g) from *V. arenaria* were extracted with CH_2Cl_2 for 30 min using a sonicator to yield 82 g of crude extract. This extract was suspended in MeOH−H_2O (9:1, v/v) and exhaustively washed with hexane and CH_2Cl_2 to yield 39.5 g (hexane phase) and 25.0 g (CH_2Cl_2 phase). The hexane phase was chromatographed over Si gel using vacuum liquid chromatography to yield six fractions: F1 (0.5 g), F2 (13.3 g), F3 (14.2 g), F4 (5.3 g), F5 (2.4 g), and F6 (3.6 g). Fraction F2 furnished the diterpene PA. Isolation and purification steps were carried out by flash chromatography (hexane−EtOAc), PTLC (Si gel, hexane−EtOAc or hexane−$CHCl_3$), and recrystallization from MeOH. The structure of the diterpene was established by comparison of the ^1H and ^{13}C NMR spectral data with those reported in the literature.[17,18]

Test Compounds. The compounds used in this study were carrageenan (Santa Cruz Biotechnology, Santa Cruz, CA, USA), L-NAME (Research Biochemicals, Natick, MA, USA), acetic acid and formaldehyde (Mallinckrodt Baker, S.A., Mexico, Mexico City), KT5823 (Calbiochem, San Diego, CA, USA), and 1*H*-(1,2,4)-oxadiazolol-(4,3-a)quinoxalin-1-one (ODQ) (Tocris Cookson, Baldwin, MO, USA); complete Freund's adjuvant (CFA), glybenclamide, and prostaglandin E_2 (PGE_2) were purchased from Sigma Chemical Company (St. Louis, MO, USA), and indomethacin was from Prodome (Campinas, SP, Brazil).

Animals. Male Swiss mice (20−25 g), from the Universidade Estadual de Londrina, Londrina, Paraná, Brazil, were used in this study. Mice were housed in standard clear plastic cages with free access to food and water and a light/dark cycle of 12:12 h and were kept at 21 °C. All behavioral testing was performed between 9 A.M. and 5 P.M. in a temperature-controlled room. Animal care and handling procedures were in accordance with the International Association for Study of Pain (IASP) guidelines and approved by the Ethics Committee of the Universidade Estadual de Londrina (process number 1531.2013.76). Every effort was made to minimize the number of animals used and their suffering.

Nociception and Writhing Response Tests. The acetic acid-induced writhing models were performed as previously described; acetic acid (0.8% v/v, diluted in saline, 10 mL/kg) or vehicle was injected into the peritoneal cavities of mice. Each mouse was placed in a large glass cylinder, and the intensity of nociceptive behavior was quantified by counting the total number of writhes occurring between 0 and 20 min after stimulus injection. The writhing response consisted of a contraction of the abdominal muscle together with a stretching of the hind limbs. The intensity of the writhing response was expressed as the cumulative writhing score over 20 min.[53]

Formalin Test. The number of paw flinches and the time spent licking the paws were determined between 0 and 30 min after intraplantar injection of 25 μL of formalin 1.5%, as previously described. The period was divided into intervals of 5 min and clearly demonstrated the presence of the first and second phases, which are characteristic of the method. Results were obtained for both the first (0−5 min) and second (10−30 min) phases.[54]

Complete Freund's Adjuvant Test. The number of paw flinches and time spent licking the stimulated paw were determined between 0 and 30 min after ipl injection of 10 μL of CFA. Results were expressed by the total number of flinches and licks performed in 30 min.[26]

Electronic Pressure-Meter Test. Mechanical hyperalgesia was assessed as previously reported.[55] In a quiet room, mice were placed in acrylic cages (12 × 10 × 17 cm) with wire grid floors, 30 min before testing. The test consisted of evoking a hind paw reflex with a hand-held force transducer (electronic anesthesiometer; Insight, Ribeirao Preto, SP, Brazil) adapted with a 0.5 mm^2 polypropylene tip. 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, and the intensity of pressure was recorded automatically. The animals were tested before (basal) and after treatment and stimuli, and the value for each interval was an average of three measurements. The results are expressed by delta (Δ) withdrawal threshold (in g), calculated by subtracting the basal mean measurements from the mean measurements obtained at 1, 3, or 5 h after ipl stimuli with the hyperalgesic agents carrageenan (300 μg/paw), CFA (10 μL/paw), and PGE_2 (100 ng/paw).

Hot Plate Test. Mice were placed in a 10 cm wide glass cylinder on a hot plate (IITC Life Science Inc., Woodland Hills, CA, USA) maintained at 55 °C. Two control latencies at least 10 min apart were determined for each mouse. The normal latency (reaction time) was 12−20 s. The latency was also evaluated 1, 3, and 5 h after test compound administration. The reaction time was scored when the animal jumped or licked its paws. A maximum latency (cutoff) was set at 30 s to avoid tissue damage.[50−52]

Myeloperoxidase Activity. Neutrophil migration to the stomach was indirectly evaluated by the MPO activity kinetic-colorimetric assay.[56] Briefly, samples were collected in 50 mM K_2PO_4 buffer (pH 6.0) containing 0.5% HTAB and were homogenized using Ultra-Turrax (IKA T10 Basic, CQA Química, Paulínea, SP, Brazil). Then the homogenates were centrifuged at 16100*g* for 2 min at 4 °C. A 15 μL amount of resulting supernatant was mixed with 200 μL of 50 mM phosphate buffer, pH 6.0, containing 0.167 mg/mL *o*-dianisidine dihydrochloride and 0.05% hydrogen peroxide and was assayed spectrophotometrically for MPO activity (450 nm, BEL SP2000UV, Photonics, São Paulo, SP, Brazil). MPO activity of samples was compared with a standard curve of neutrophils and presented as MPO activity (number of neutrophils/mg of tissue).

Cytokine Measurement. Mice were treated with vehicle or **1** (10 mg/kg, po) 30 min before carrageenan (300 μg/paw) stimulus. Three hours after the injection of carrageenan, mice were euthanized, and the skin tissues were removed from the injected and control paws (saline). The samples were homogenized in 500 μL of buffer containing protease inhibitors, and TNF-α, IL-1β, IL-33, and IL-10 levels were determined as described previously[2] by an enzyme-linked immunosorbent assay (ELISA) using eBioscience kits. The results are expressed as pg of cytokine/100 mg of tissue. As a control, the concentrations of these cytokines were determined in animals injected with saline.

NF-κB Activity. The assessment of total and phosphorylated NF-κB production was performed by ELISA using Cell Signaling Technology kits. Mice were treated with vehicle or **1** (10 mg/kg, po) 30 min before carrageenan (300 μg/paw) stimulus. Three hours after the injection of carrageenan, mice were euthanized, and the skin tissues were removed and homogenized in lysis buffer. Lysates were centrifuged (3000 rpm/10 min, 4 °C), and the supernatant was used to assess the levels of total and phosphorylated NF-κB by ELISA following the manufacturer's instructions. The results were obtained by comparing the optical density of samples and the samples' weight.

Hepatic Toxicity and Gastric Inflammation. Aspartate aminotransferase and alanine aminotransferase plasmatic levels and MPO activity in the stomach were used as indicators of hepatic toxicity and gastric inflammation, respectively.[26] Mice treated daily for 7 days with **1** (10 mg/kg, po), vehicle (DMSO 2% diluted in saline or Tris/HCl buffer, pH 8.0), or indomethacin (2.5 mg/kg) were terminally anesthetized, and blood and stomach samples were collected. The

blood samples were collected in heparinized tubes and centrifuged (200*g*, 10 min, 4 °C). AST and ALT plasma levels were determined by using a diagnostic kit from Labtest according to the manufacturer's instructions. The MPO activity in stomach samples was determined and is described in the next section.

Measurement of Motor Performance. In order to discard possible nonspecific muscle relaxant or sedative effects of **1**, mice motor performance was evaluated using the rota-rod test. The apparatus consisted of a bar with a diameter of 2.5 cm, subdivided into four compartments by disks 25 cm in diameter (Ugo Basile, model 7600). The bar rotated at a constant speed of 22 rotations per min. The animals were selected 24 h previously by eliminating those mice that did not remain on the bar for two consecutive periods of 180 s. Animals were treated with vehicle (DMSO 2% in saline) or **1** (10 mg/kg, po), and testing was performed 1.5, 3.5, and 5.5 h after treatment. The cutoff time used was 180 s.[49]

Statistical Analysis. Results are presented as means ± SEM of experiments made on five (Figure 5), six (Figures 1−4, 6, and 7), or eight (Figure 8) animals per group and are representative of two separate experiments. Two-way analysis of variance (ANOVA) was used to compare the groups and doses at all times (curves) when the hyperalgesic responses were measured at different times after the administration or enforcement of the stimuli. The factors analyzed were treatment, time, and time versus treatment interaction. When there was a significant time versus treatment interaction, one-way ANOVA followed by Tukey's *t* test was performed on each occasion. When the hyperalgesic responses were measured once after the administration or enforcement of the stimuli, the difference between responses was evaluated by one-way ANOVA followed by Tukey's *t*-test. Statistical differences were considered to be significant at $p < 0.05$.

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Notes

The authors declare no competing financial interest.

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