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The Quassinoid Isobrucein B Reduces Inflammatory Hyperalgesia and Cytokine Production by Post-transcriptional Modulation

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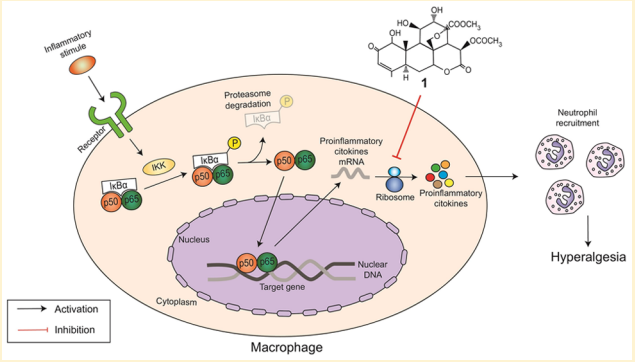
Supporting Information

ABSTRACT: Isobrucein B (**1**) is a quassinoid isolated from the Amazonian medicinal plant *Picrolemma sprucei*. Herein we investigate the anti-inflammatory and antihyperalgesic effects of this quassinoid. Isobrucein B (**1**) (0.5−5 mg/kg) inhibited carrageenan-induced inflammatory hyperalgesia in mice in a dose-dependent manner. Reduced hyperalgesia was associated with reduction in both neutrophil migration and pronociceptive cytokine production. Pretreatment with **1** inhibited in vitro production/release of cytokines TNF, IL-1β, and KC/CXCL1 by lipopolysaccharide-stimulated macrophages. To investigate its molecular mechanism, RAW 264.7 macrophages with a luciferase reporter gene controlled by the NF-κB promoter were used (RAW 264.7-Luc). Quassinoid **1** reduced the luminescence emission by RAW 264.7-Luc stimulated by different compounds. Unexpectedly, NF-κB translocation to macrophage nuclei was not inhibited by **1** when evaluated by Western blotting and immunofluorescence. Furthermore, quassinoid **1** did not change the levels of TNF mRNA transcription in stimulated macrophages, suggesting post-transcriptional modulation. In addition, constitutive expression of luciferase in RAW 264.7 cells transiently transfected with a plasmid containing a universal promoter was inhibited by **1**. Thus, isobrucein B (**1**) displays anti-inflammatory and antihyperalgesic activities by nonselective post-transcriptional modulation, resulting in decreased production/release of pro-inflammatory cytokines and neutrophil migration.



Inflammation can be initiated by tissue injury or infection. Enhancement of pain sensitivity (hyperalgesia) is one of the most common symptoms of inflammatory processes. This symptom is mainly caused by the sensitization of primary nociceptive neurons, which is triggered by several mediators released during the inflammatory response.[1,2] Pro-inflammatory cytokines are some of the most important mediators involved in inflammatory hyperalgesia. For instance, a cascade of cytokines initiated by the release of tumor necrosis factor-α (TNF) and keratinocyte-derived chemokine (KC/CXCL1) plays a crucial role in the induction of inflammatory hyperalgesia.[3] In this cascade of pronociceptive mediators, TNF stimulates interleukin-1β (IL-1β) production, which in turn stimulates the production of prostaglandins. Besides triggering the IL-1β/prostanoid pathway, KC/CXCL1 is also able to stimulate the sympathomimetic component of inflammatory hyperalgesia.[3] Importantly, the peripheral pronociceptive action of these cytokines in acute inflammation depends on neutrophil recruitment.[4] The inhibition of pro-inflammatory cytokines/chemokine action/production or neutrophil migration may reduce inflammatory hyperalgesia and is a potential target for the development of new antihyperalgesic drugs.[1−5] In fact, some cytokine-targeting immunobiologicals reduce pain sensitivity and are already used in the treatment of inflammatory diseases.[6−8] However, immunobiologicals have cost and pharmacokinetic parameters as limitations. There is still a need to develop drugs that reduce the release/action of these cytokines. Furthermore, natural products represent an important source of structurally diverse compounds for the development of anti-inflammatory, analgesic, and other classes of drugs.[9,10]

Isobrucein B (**1**) is a quassinoid natural product that can be isolated from root, stem, and leaf extracts of *Picrolemma sprucei* Hook. f. (Simaroubaceae).[11,12] Quassinoids have a wide variety of biological activities in vitro and/or in vivo, including antitumor, antimalarial, antiviral, anti-inflammatory, insecticidal, amoebicidal, anthelmintic, and antiulcer activities.[13−17] Infusions and other preparations of the roots, stems, and leaves of *P. sprucei* are used traditionally by the peoples of the Amazon region to treat gastropathies, malaria, and helminth infections.[11,13,14] Recently, it was demonstrated that mice pretreated with **1** exhibited reduced gastric damage after acute administration of a nonsteroidal anti-inflammatory drug.[17] In addition, gastroprotective action of **1** was associated with a reduction in the production of pro-inflammatory cytokines,

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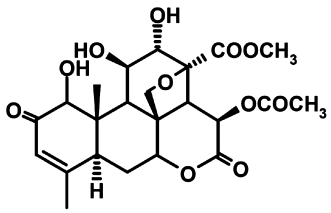
suggesting that **1** might have anti-inflammatory activity.[17] Furthermore, cytokines play an important role in the induction of inflammatory hyperalgesia. Thus, we asked whether **1** might also have antihyperalgesic effects. Herein, the potential effect of isobrucein B (**1**) upon inflammatory hyperalgesia was investigated. In addition, its molecular mechanism on the reduction of cytokine production was clarified.



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RESULTS AND DISCUSSION

Decreased production of pro-inflammatory cytokines and neutrophil migration induced by isobrucein B (**1**) in a model of gastritis[17] prompted us to investigate whether **1** could reduce inflammatory pain, which is mainly dependent on pro-inflammatory cytokines and neutrophil migration.[3,4] Thus, the present study demonstrates that **1** is able to reduce inflammatory hyperalgesia associated with a decrease in the production of pronociceptive cytokines (IL-1β and KC/CXCL1) and neutrophil migration. The antihyperalgesic effect of **1** was evaluated in a carrageenan-induced acute inflammatory hyperalgesia model. Pretreatment (30 min prior) of mice with **1** reduced carrageenan-induced mechanical inflammatory hyperalgesia in a dose-dependent manner (Figure 1A). A dose of 5 mg/kg of **1** abolished mechanical hyperalgesia. On the other hand, treatment of mice with **1** does not alter the baseline thermal nociceptive threshold. Altered thermal nociceptive threshold was clearly observed when morphine was administered (Figure 1B). This result is consistent with the existence of an antihyperalgesic effect that is distinct from that of opioids.

Since pro-inflammatory cytokines (TNF, IL-1β, and KC/CXCL1) play a crucial role in the induction of acute inflammatory hyperalgesia,[3] the next step was to evaluate the effect of **1** on the production/release of these cytokines in carrageenan-induced inflammation. Mice were pretreated with the effective antihyperalgesic dose of **1** (5 mg/kg), and 3 h after carrageenan paw injection, the levels of TNF, IL-1β, and KC/CXCL1 were evaluated in the inflamed tissue. Pretreatment with **1** inhibited the increase of local production/release of IL-1β (Figure 2B) and KC/CXCL1 (Figure 2C), but it did not prevent increases in TNF levels after carrageenan-induced paw inflammation (Figure 2A).

The pronociceptive effect of cytokines depends on neutrophil recruitment.[4] In this regard, pretreatment of mice with **1** was also able to inhibit neutrophil migration toward mice paws after carrageenan injection (Figure 2D). The strong inhibition of neutrophil migration, reduced production of pro-inflammatory cytokines IL-1β and KC/CXCL1, and unchanged TNF levels, associated herein with antihyperalgesia, are also associated with the mechanism of **1** on reduction of indomethacin-induced gastric lesions published previously by our group.[17] IL-1β and KC/CXCL1 are known to have an important role in the development of hyperalgesia through the production/release of prostanoids and sympathetic amines, respectively.[3] Thus, the powerful antihyperalgesic effects of **1** are associated with reduced levels of these cytokines and inhibition of neutrophil migration.[4]

In an attempt to identify the molecular mechanism by which **1** downregulates cytokine production, macrophages in culture were used. Macrophages are one of the main sources of pro-inflammatory cytokines and chemokines in tissues after administration of inflammatory stimuli.[18,19] Then, the next experiments were conducted in primary cultures of these cells harvested from the peritoneal cavity of mice or an immortalized murine macrophage lineage, RAW 264.7. Preincubation of primary murine macrophages with **1** inhibited the production/release of TNF, IL-1β, and KC/CXCL1 induced by lipopolysaccharide (LPS) stimulation in a concentration-dependent manner in the 0.1−10 μM concentration range (Figure 3A−C). Dexamethasone was used as positive control. Importantly, the inhibitory effect of **1** on the production of pro-inflammatory cytokines by macrophages was not associated with a reduction in cell viability, which was evaluated through trypan blue exclusion (Figure S1, Supporting Information),

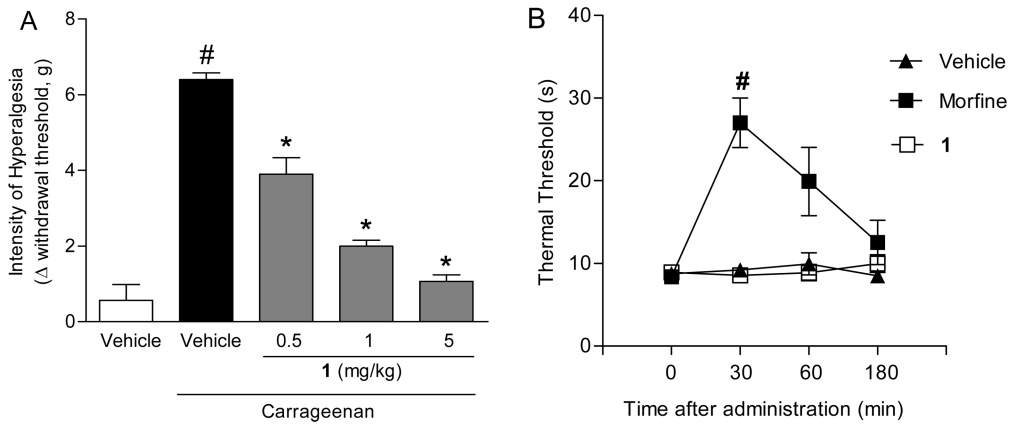

Figure 1. Isobrucein B (**1**) reduces carrageenan-induced inflammatory hyperalgesia, but had no effect on thermal nociception. (A) Mice were pretreated with vehicle or **1** subcutaneously (sc) 1 h before intraplantar injection of carrageenan. Mechanical hyperalgesia was assessed 3 h after carrageenan injection. (B) To evaluate thermal nociception, mice were treated with vehicle, **1** (1 mg/kg, sc), or morphine (8 mg/kg, sc) and immediately submitted to the hot plate test. Data are the means ± SEM (n = 6). $^{\#}p < 0.05$ when compared to the vehicle group; $^{*}p < 0.05$ when compared to the group treated with carrageenan/vehicle.

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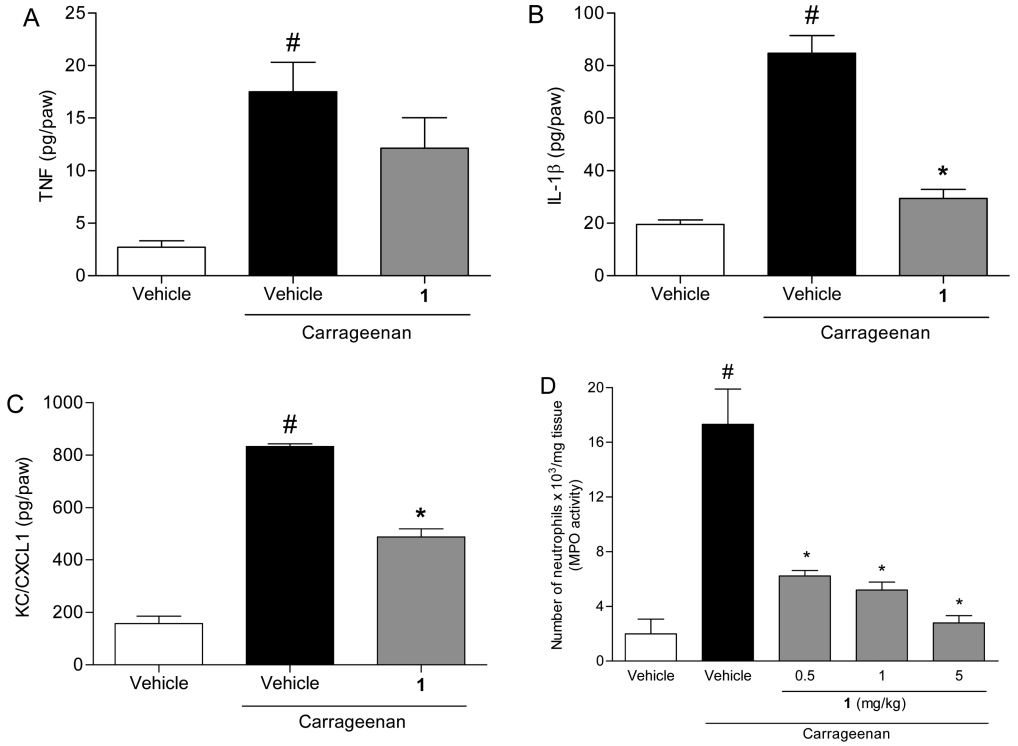

Figure 2. Effect of isobrucein B (**1**) in carrageenan-induced pronociceptive cytokine production and neutrophil migration. (A–C) Mice were pretreated sc with vehicle or **1** (5 mg/kg) 1 h before intraplantar injection of carrageenan, and 3 h later plantar tissues were collected for quantification of the levels of (A) TNF, (B) IL-1β, and (C) KC/CXCL1 by ELISA. (D) Mice were pretreated sc with vehicle or **1** (0.5, 1, and 5 mg/kg) 1 h before intraplantar injection of carrageenan. Three hours after carrageenan injection plantar tissues were collected for quantification of neutrophil migration by MPO activity. Data are the means ± SEM (n = 5). $^{\#}p < 0.05$ when compared to the vehicle group; $^{*}p < 0.05$ when compared to the group treated with carrageenan/vehicle.

MTT reduction (Figure 4A), and lactate dehydrogenase (LDH) activity (Figure 4B).

The production of pro-inflammatory cytokines, such as TNF, IL-1β, and KC/CXCL1, by LPS-stimulated macrophages is mainly dependent on the NF-κB signaling pathway.[20−23] Similarly, another quassinoid, brucein D, inhibits the constitutively activated NF-κB signaling pathway[24] in pancreatic adenocarcinoma PANC-1 cells by decreasing IκB-α and p65 phosphorylation and NF-κB DNA-binding.[25] Furthermore, quassinoids from *Eurycoma longifolia* inhibit luciferase activity in TNF-stimulated HEK293/NF-κB-luciferase cells,[26] suggesting an inhibitory effect of these quassinoids on NF-κB signaling. Thus, the inhibitory effect of **1** on pro-inflammatory cytokine production could be due to down-modulation of the NF-κB signaling pathway. Therefore, RAW 264.7 macrophages stably bearing the luciferase gene-containing vector controlled by an NF-κB-activated promoter were used to test this hypothesis. Four different compounds were used to induce NF-κB activation and signaling in macrophages. Remarkably, preincubation of RAW 264.7 macrophages with **1** promoted concentration-dependent reduction of luminescence emission induced by LPS (toll-like receptor-4 [TLR4] agonist), peptidoglycan (agonist of TLR2 and nucleotide-binding oligomerization domain-containing protein 2 [NOD2]), and TNF or phorbol myristate acetate (PMA; activator of protein kinase-C) (Figure 5A, B, C, and D, respectively).[27−30] Dexamethasone was used as a control for reduced luciferase expression. Interestingly, incubation of RAW 264.7 macrophages with **1** (10 μM) after induction of enzyme expression by LPS (4 h after LPS incubation) did not change the luminescence, indicating **1** is not interfering directly with the catalytic activity of luciferase (Figure S2, Supporting Information). **1** probably modulates luciferase expression through the inhibition of NF-κB signaling pathways.

Nevertheless, we further analyzed the effect of **1** on two important molecular steps in the NF-κB signaling pathway: IκB-α degradation and p65 (a subunit of NF-κB) translocation to the nucleus. However, it was found that **1** failed to reduce IκB-α degradation (Figure 6A), and using two different approaches (Western blotting and confocal microscopy), **1** did not change the translocation of the p65 NF-κB subunit to the nucleus of RAW 264.7 macrophages induced by LPS (Figure 6B and C). Pyrrolidine dithiocarbamate, a known inhibitor of NF-κB activation,[31] was used as the control. These results indicate that **1** could be affecting NF-κB signaling at transcriptional or post-transcriptional levels.

Continuing the reasoning, the effect of **1** on the transcriptional activity of NF-κB was investigated by measuring the TNF mRNA levels in macrophages pretreated with vehicle, **1** (10 μM), or dexamethasone (1 mM) and stimulated by LPS. Surprisingly, isobrucein B (**1**) did not reduce the increase in the TNF mRNA expression in stimulated macrophages, whereas the increase in TNF mRNA expression was reduced by dexamethasone (Figure 7), although inhibiting the production of this cytokine (Figure 3A). These results are in accordance with the hypothesis that **1** may therefore act through post-transcriptional mechanisms.

These apparent contradictory effects of **1** compared to other quassinoids, such as brucein D[25] and quassinoids isolated from *Eurycoma longifolia*,[26] are very probably dependent upon

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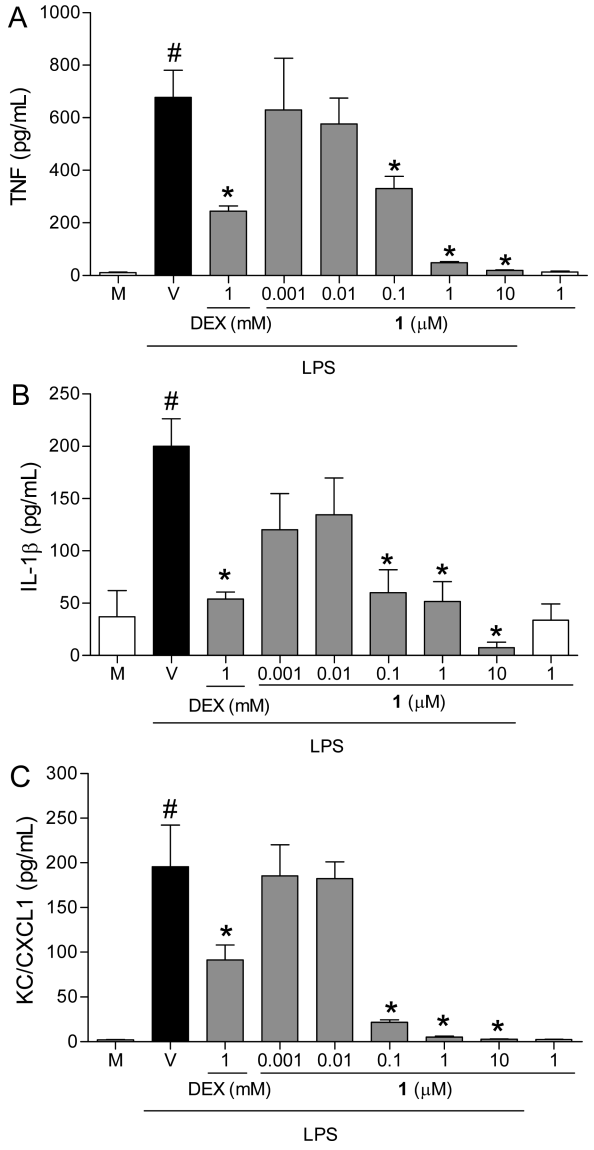

Figure 3. Isobrucein B (**1**) inhibits pro-inflammatory cytokine production by macrophages in vitro. Peritoneal macrophages in culture medium (M) were pretreated with vehicle (V), **1** (0.001−10 μM), or dexamethasone (DEX; 1 mM) 30 min before incubation with LPS (1 μg/mL). The supernatants were removed 4 h after LPS stimulation for quantification of the levels of (A) TNF, (B) IL-1β, and (C) KC/CXCL1 by ELISA. Data express means ± SEM (n = 4). $^{\#}p$ < 0.05 when compared to the vehicle group; $^{*}p$ < 0.05 when compared to the group treated with LPS/vehicle.

differences in the molecular structures. Alternatively, the use of different cell systems, i.e., macrophage versus PANC-1 cells, the latter of which has NF-κB constitutively activated by upstream signaling cascades involving Ras and mitogen-activated protein kinases (MAPK), could also explain these differences.[24] For instance, brucein D could be inhibiting NF-κB-activating upstream kinases (e.g., Ras and MAPK) and consequently promoting an indirect reduction in p65 and IκB-α phosphorylation and NF-κB activation. Also, it seems that in macrophages activated by LPS, MAPK and Ras kinase are not crucial for NF-κB activation.[27−30,32] For quassinoids from *Eurycoma longifolia*, the conclusions were based only on an NF-κB reporter assay after TNF stimulation.[26]

Since **1** might not act by blocking transcriptional activity of NF-κB but could instead be inhibiting the production/release of NF-κB-dependent cytokines, the next hypothesis was that **1** activities could likely be mediated by modulation of post-transcriptional mechanisms. To confirm this hypothesis, we used RAW 264.7 cells transfected with the luciferase gene vector controlled by a phosphoglycerate kinase promoter (PKG), a nonviral universal promoter, a region that promotes the recruitment of transcription factors nonspecifically. Interestingly, quassinoid **1** inhibits the luminescence emission induced by nonspecific transcription factors (Figure 8). Luminescence emission was also inhibited by cycloheximide, a well-characterized protein synthesis inhibitor,[33] but not by dexamethasone or pyrrolidine dithiocarbamate. Thus, **1** might be acting nonspecifically through modulation of a post-transcriptional mechanism, probably inhibition of protein synthesis nonspecifically. Thus, we are demonstrating in this work that the NF-κB reporter assay may provide false positive results, due to drugs acting on post-transcriptional levels and leading to inhibition of luminescence emission without modulation of NF-κB signaling.

Interestingly, in vivo production of IL-1β and KC/CXCL1 but not of TNF could be inhibited by **1**; however it may reduce all these cytokines in vitro. In this context, as observed with **1**, the inhibitor of protein synthesis, cycloheximide, did not change in vivo production of TNF (Figure S3A, Supporting Information) triggered by carrageenan, despite the large decrease in IL-1β (Figure S3B, Supporting Information) production in the same paw. The TNF released during carrageenan-induced paw inflammation seems to be preformed, which could explain why **1** was not able to affect the level of this cytokine. Similarly, preformed TNF released by mast cells has been reported in some experimental models of inflammation. For instance, in animal models of immune complex induced peritonitis, the release of TNF occurs in two peaks. The first peak (5 to 15 min) is characterized by preformed TNF released by mast cells, whereas the second peak (4 to 8 h) is caused by newly synthesized TNF.[34] In addition, a similar role of mast cells on TNF release has been demonstrated during the genesis of dermal inflammation in human skin,[35] supporting our findings. Furthermore, membrane-bound TNF can be cleaved and released after cell activation.[36,37]

Many quassinoids, including **1**, share a skeletal molecular structure that enables the inhibition of eukaryotic protein synthesis.[38,39] Furthermore, quassinoids exhibiting structures similar to **1**, such as bruceatin, are able to bind to the peptidyl transferase center at the ribosomal level, thus blocking the initiation of peptide formation and consequently protein synthesis.[40,41] Therefore, it is plausible to suggest that **1** may affect protein synthesis through a similar mechanism of action.

Although post-transcriptional modulation seems to be the preeminent effect of **1**, this effect could also be mediated through inhibition of other nonevaluated transcription factors, such as activator protein 1 (AP-1). Indeed, Beutler et al.[42] showed that some quassinoids inhibit AP-1 activity without affecting protein synthesis at determined concentrations. However, there are important structural differences between these quassinoids and **1**, such as a C-17 to C-13 bridge, which is not present in **1** and seems be crucial to quassinoid inhibition of AP-1.[36,38]

Herein, the mechanism of action of **1** on the reduction of inflammatory hyperalgesia is linked to inhibition of IL-1β, KC/CXCL1, neutrophil migration, and probably inhibition of the

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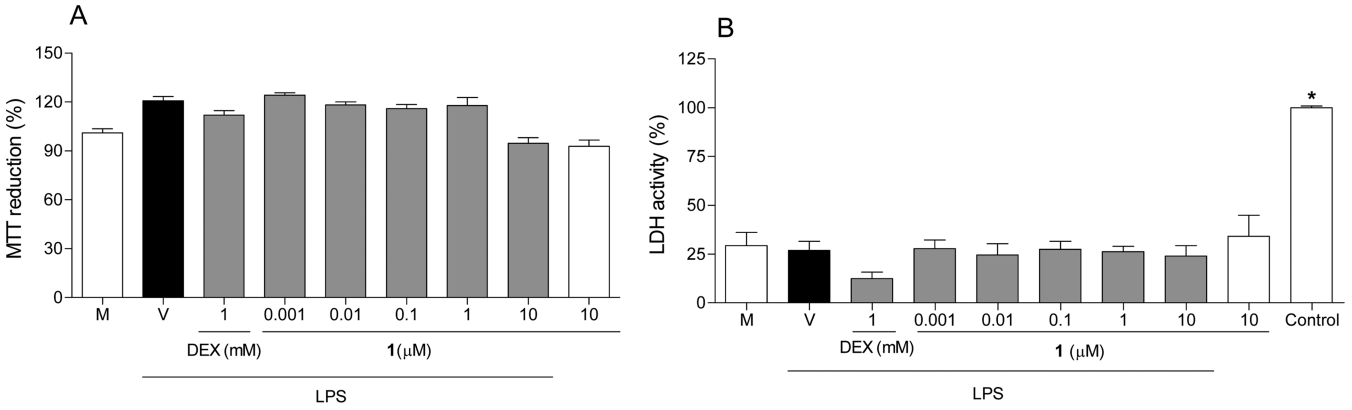

Figure 4. Isobrucein B (**1**) has no effect on cell viability. Peritoneal macrophages in culture medium (M) were pretreated with vehicle (V), **1** (0.001−10 μM), or dexamethasone (DEX) (1 mM) 30 min before incubation with LPS (1 μg/mL). Adherent cells and supernatants were used for the quantification of cell viability thought the (A) MTT assay and (B) LDH activity assay, respectively. Data are the means ± SEM (n = 4, 5); $^{\#}p$ < 0.05 when compared to the vehicle group; $^{*}p$ < 0.05 when compared to the group treated with LPS/vehicle.

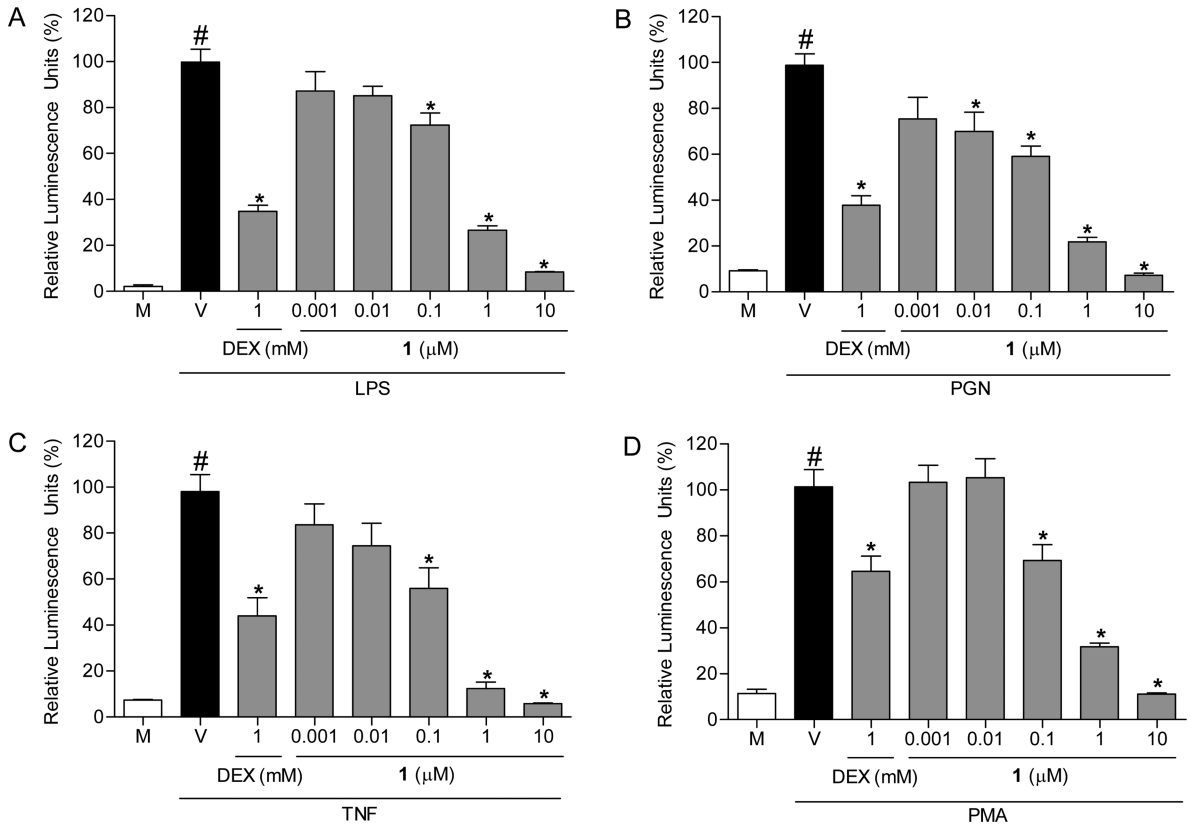

Figure 5. Isobrucein B (**1**) inhibits luminescence emission on macrophages RAW 264.7-Luc NF-κB-luciferase reporter. Cells in culture medium (M) were pretreated with vehicle (V), **1** (0.001−10 μM), or dexamethasone (DEX; 1 mM) 30 min before the addition of following stimuli: (A) LPS (1 μg/mL), (B) PGN (10 μg/mL), (C) TNF (10 ng/mL), or (D) PMA (1 μg/mL). At 4 h after stimuli incubation, cells were lysed and luminescence emission was determined as described in the Experimental Section. The results are expressed as relative luminescence units to the respective stimuli normalized as 100% (n = 4, 5). Data are the means ± SEM; $^{\#}p$ < 0.05 when compared to the vehicle group; $^{*}p$ < 0.05 when compared to the group treated with stimulus/vehicle.

synthesis of other inflammatory protein mediators. While **1** reduces inflammatory hyperalgesia, it does so through a mechanism involving nonspecific inhibition of protein synthesis that could limit its therapeutic usefulness. However, further studies regarding the effect of **1** on tumor cells are encouraged, and this natural compound could be further explored as a cancer chemotherapy agent.

EXPERIMENTAL SECTION

General Experimental Procedures. Dexamethasone, pyrrolidine dithiocarbamate, morphine, and cycloheximide were purchased from Sigma (St. Louis, MO, USA). Dexamethasone and pyrrolidine dithiocarbamate were dissolved in DMSO (Sigma), and the resulting solution was then diluted in RPMI-1640 medium (final concentration of DMSO in this solution was <0.5%), prepared as described in the cell culture section. Cycloheximide was diluted in DMSO and then in RPMI-1640 medium for in vitro experiments or saline for in vivo

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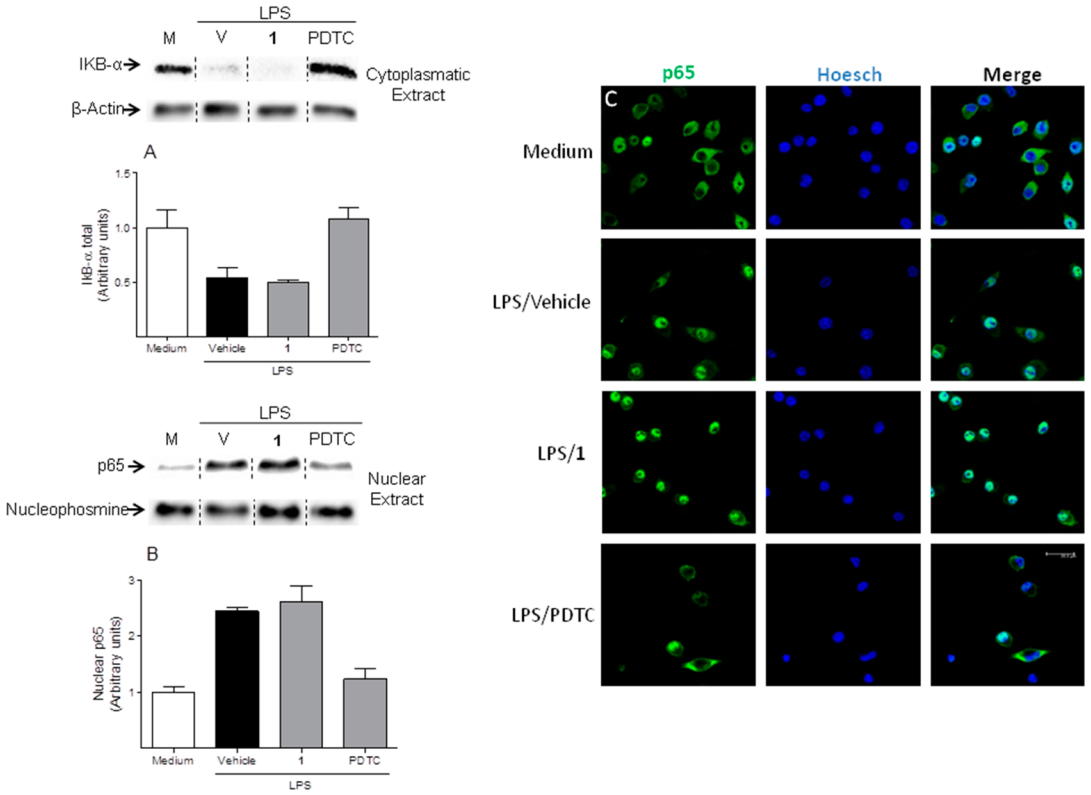

Figure 6. Isobrucein B (**1**) has no effect on IκB-α degradation or NF-κB (p65) translocation to the nucleus. RAW 264.7 macrophages were preincubated with medium (M), vehicle (V), **1** (10 μM), or pyrrolidine dithiocarbamate (PDTC; 500 μM) 30 min before the addition of LPS ((A and B) 1 μg/mL). (A and B) After 30 min incubation under stimulation, cells were lysed in order to separate the cytoplasmic and nuclear contents. Bands exhibited are representative of the group. (A) In the cytoplasmic extract, IκB-α and β-actin proteins were evaluated. (B) Proteins p65 and nucleophosmin were evaluated in nuclear extracts. Bars express the normalized value compared to the medium control group. (C) Cells were fixed 30 min after LPS (100 ng/mL) and then stained with p65/Alexa-488 (green) and nucleus/Hoesch 33342 (blue) ($n \geq 2$). $^{\#}p < 0.05$ compared to vehicle group; $^{*}p < 0.05$ compared with the group stimulated with LPS/vehicle.

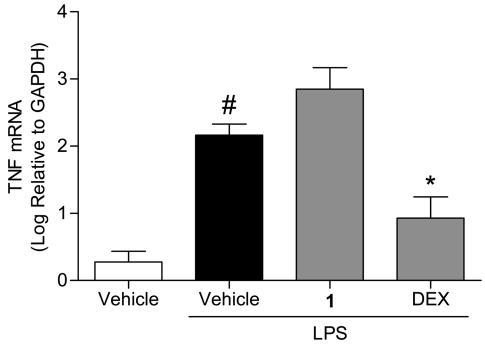

Figure 7. Isobrucein B (**1**) does not inhibit TNF mRNA transcription. RAW 264.7 were stimulated with LPS (1 μg/mL) 30 min after treatment with vehicle, **1** (10 μM), or dexamethasone (DEX; 1 mM). Cells were lysed 2 h after LPS stimulation. The quantification of TNF mRNA was realized by RT PCR. To allow the use of parametric statistical test, the results were transformed in their respective logarithm ($y = \log y$). $^{\#}p < 0.05$ compared to vehicle group; $^{*}p < 0.05$ compared with vehicle/LPS group.

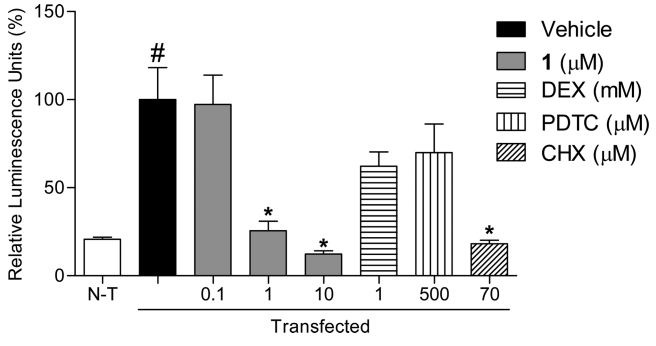

Figure 8. Isobrucein B (**1**) impaired constitutive luciferase synthesis. RAW 264.7 macrophages were nontransfected (N-T) or transfected with luciferase plasmid containing the universal promoter PKG, which promotes constitutive expression of luciferase. RAW 264.7 transfected cells were incubated with vehicle, **1** (0.1−10 μM), dexamethasone (DEX; 1 mM), pyrrolidine dithiocarbamate (PDTC; 500 μM), or cycloheximide (CHX; 70 μM) 6 h after the transfection medium was added. The luminescence was evaluated 6 h after incubation with the drugs. The results are expressed as relative luminescence units to the transfected/vehicle group as 100% ($n \geq 3$). $^{\#}p < 0.05$ compared to vehicle group; $^{*}p < 0.05$ compared with the transfected/vehicle group.

experiments (DMSO ≤ 0.5%). Carrageenan was obtained from FMC (Philadelphia, PA, USA) and dissolved in phosphate buffer.

Plant Material. Isobrucein B (**1**) was isolated from the stems and roots of *Picrolemma sprucei* Hook f. collected in April 2003 in Amazonas State, Brazil. The collected material was identified by Dr. W. Thomas (New York Botanical Garden, New York, NY, USA). Voucher specimens (Silva 5729 and 5730) were deposited at the Herbarium of the Federal University of Amazonas (UFAM), Manaus, Amazonas State, Brazil.

Extraction and Isolation of Isobrucein B (1). A detailed procedure for extraction and isolation of **1** on a gram scale has been previously published.[44] Briefly, this procedure consists of pilot-scale

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degreasing by Soxhlet extraction with hexanes of the dried, ground roots and stems of *P. sprucei*. The plant materials were further extracted with water in a Soxhlet apparatus. The water extracts were partially concentrated and liquid−liquid extracted with chloroform. The chloroform extract was totally evaporated. IsoB (**1**) was obtained from the chloroform extracts by fractional recrystallization from water−acetone. The identity of **1** was ascertained from NMR and other spectra. Further corroboration of the structure of **1** was obtained by preparation and characterization of the 1,12-diacetyl isobrucein B derivative by NMR and HRMS techniques.[13,43,44] As a purity check prior to pharmacological evaluation, high-performance liquid chromatography (HPLC) was performed using 10:90 acetonitrile−water, with a linear gradient (20 min) to 75:25 acetonitrile−water and isocratic until the end of the run as mobile phase, a reversed phase (ODS) analytical column, sample injection volume of 5 μL, flow rate of 1.5 mL/min, and UV detection at λ_{max} 243 and 254 nm. Only one compound was detected in the chromatographic analyses. From the NMR and HPLC analyses, a purity of >98% was established for **1** used in this work. HPLC chromatograms and NMR spectral data are available upon request. **1** was dissolved in DMSO and then diluted in saline solution or in RPMI-1640 culture medium for in vivo or in vitro experiments, respectively. The final concentrations of DMSO in these solutions were ≤1% or ≤0.5%, respectively.

Animals. Prior approval for this work was obtained from the Animal Ethics Committee of the Ribeirão Preto Medical School at the University of São Paulo (protocol number 077/2012). The experiments were performed on male C57BL/6 mice weighing 20−25 g. The animals were maintained in temperature-controlled rooms (23−25 °C, light−dark cycle of 12 h) and given water and food ad libitum. Animal care and handling procedures were performed in accordance with the guidelines of the International Association for Study of Pain.

Determination of Mechanical Inflammatory Hyperalgesia: Electronic Von Frey Test. Mechanical hyperalgesia was tested in mice as reported previously.[45] Briefly, mice were placed in acrylic cages (12 × 10 × 17 cm) with wire grid floors in a quiet room 15−30 min before testing commenced. The test consisted of evoking a hindpaw flexion reflex with a hand-held force transducer (electronic esthesiometer, IITC Life Science, Woodland Hills, CA, USA) adapted with a 0.5 mm^2 polypropylene tip. The investigator was trained to apply the tip perpendicularly to the central area of the plantar hind paw with a gradual increase in pressure. The gradual increase in pressure was manually performed in blinded experiments. The upper pressure limit was 15 g. The end point was characterized by the animal withdrawing its paw followed by clear flinching movements. The intensity of the pressure that elicited paw withdrawal was automatically recorded. The final value for the response was obtained by averaging three measurements. The animals were tested before and after treatments, such that the experimenter who evaluated hyperalgesia had no knowledge of the treatment received by the animals. The results are expressed as the delta (Δ) withdrawal threshold (in g) calculated by subtracting the zero-time mean measurements from the mean measurements at 3 h after intraplantar (ipl) administration of carrageenan (100 μg/25 μL). The animals were previously treated with **1** subcutaneously (sc) (0.5, 1.0, and 5.0 mg/kg) or vehicle 1 h before carrageenan injection.

Hot-Plate Test. As a means to investigate whether **1** presents opioid-like antinociceptive activity, the effect of **1** was evaluated in the hot-plate test. The hot-plate test consists of exposing mice to a 10 cm wide, 51 °C metal plate in a glass cylinder. The temperature threshold is reached when the animal suddenly withdraws or licks its paws and is based on time in seconds of exposure to the heated plate. For this test, the thermal nociceptive threshold was recorded for mice treated with saline, **1** (1 mg/kg), or morphine (8 mg/kg). At indicated time points after drug injection, thermal thresholds were recorded again. The results are expressed as thermal threshold in seconds. All experiments were performed blind so that the experimenter that administered the drugs was not the same person who assessed the nociceptive behavior.

Myeloperoxidase (MPO) Activity Assay. The tissue myeloperoxidase activity was used as a way to indirectly quantify the local accumulation of neutrophils, as previously described.[4] For this test, the neutrophil migration was induced by carrageenan (100 μg/25 μL, ipl), and then plantar tissue of treated mice was removed and transferred individually into tubes containing 50 mM K_2HPO_4 and 0.5% hexadecyl trimethylammonium bromide pH 6.0 buffer. The samples were ground and homogenized using a Polytron mixer (PT3100) and then centrifuged at 16000*g* for 4 min. Then, 10 μL of the resulting supernatant was added to 200 μL of 50 mM K_2HPO_4 buffer, pH 6.0, containing 0.167 mg/mL *o*-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. The MPO activity was measured by spectrophotometry (absorbance) at a wavelength of 450 nm. The activity obtained was compared to a standard curve of neutrophils obtained from the peritoneal cavity of mice 6 h after administration of sterile 3% sodium thioglycolate in phosphate buffer (PBS). MPO activity is equivalent to the number of neutrophils per milligram of tissue.

Peritoneal Macrophage Culture. Macrophages were obtained from mice peritoneal cavities 4 days after intraperitoneal (ip) administration of 2 mL of 3% sodium thioglycolate solution. Before collection, mice were sedated with 2% isoflurane in an anesthesia chamber and then euthanized. Peritoneal cells were harvested by washing the peritoneal cavity with RPMI-1640 medium. After centrifugation, the supernatant was discarded, and cells were resuspended in supplemented RPMI-1640 medium. The concentration of peritoneal macrophages was adjusted to 1×10^6 cells/mL, and 2×10^5 cells were added to each well in a 96-well plate, incubated at 37 °C in 5% CO_2 overnight.[46] Before the experiments, the supernatant liquids were replaced to remove nonadherent cells. Then, macrophages were pretreated with vehicle, **1** (0.001 to 10 μM), or dexamethasone (1 mM) 30 min prior to addition of the stimulus LPS (derived from *E. coli*; Sigma) to a final concentration of 1 μg/mL. After 4 h of stimulation, the supernatant liquids were removed and stored at −70 °C until quantification of cytokines could be performed.

Enzyme Linked Immunosorbent Assay (ELISA). Quantification of TNF, IL-1β, and CXCL1/KC was performed using ELISA assays, following the manufacturer's instructions for the Duo Set Kit (R&D Systems, Minneapolis, MN, USA). The results are expressed as concentrations (pg/mL or pg/paw).

Cell Viability. To assess the cytotoxic potential of **1**, peritoneal macrophages in culture were treated using the same protocol described above for the assay and quantification of cytokines released in vitro. Three methods were used to detect cell viability: (1) exclusion of trypan blue, (2) reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay), and (3) activity of extracellular lactate dehydrogenase.

The MTT assay was performed by adapting the procedure previously described by Mosmann.[47] After the incubation period, supernatants were removed and the cells were incubated with MTT solution (200 μL; 1 mg/mL) (tetrazolium salt; Sigma) in RPMI-1640 medium for 2 h. Then, the solution was removed and intracellular reduced MTT (formazan) was dissolved in 200 μL of DMSO for 30 min with stirring. For analysis, 100 μL of the samples was transferred to a 96-well plate and quantified by spectrophotometric absorbance at a wavelength of 590 nm. Results are expressed as percentage reduction compared to the control group (vehicle).

To detect the activity of LDH, the Citotoxicity Detection Plus LDH kit (Roche Life Science, Mannheim, BW, Germany) was used following the manufacturer's instructions. Readings were performed at 492 nm. For the control group, cultured cells were incubated with lysis buffer for 5 min. The LDH activity detected in this group was represented as 100% (maximum cell death).

Luciferase-Nuclear Factor Kappa B (NF-κB) Reporter Assay. RAW 264.7 macrophages that stably bear the luciferase reporter gene controlled by an NF-κB-sensitive promoter (pNF-κB-Luc) were used.[48] Before each experiment, the supernatants were replaced to remove nonadherent cells. Cultured cells were pretreated with **1** at different concentrations (0.001 to 10 μM) or dexamethasone (1 mM) 30 min prior to addition of different stimuli: peptidoglycan (PGN; 10 μg/mL; Sigma), lipopolysaccharide (LPS; 1 μg/mL; Sigma), TNF (10 ng/mL), or phorbol myristate (PMA; 1 μg/mL; Sigma). For analysis, cells were lysed with 50 μL of TNT lysis buffer at 4 °C for 20 min. Lysates (10 μL) were transferred to an opaque white plate and then

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incubated with 25 μL of Luciferase Assay Reagent containing luciferin (Promega, Madison, WI, USA). Results are expressed as luminescence emission that is released after luciferin oxidation by induced luciferase. Luminescence emission was measured using a luminometer (FlexStation 3, Molecular Devices, Sunnyvale, CA, USA).

Quantitative Real-Time Polymerase Chain Reaction (RT-PCR). RAW 264.7 macrophages (1.5×10^6 cells/well), cultured in supplemented RPMI-1640 medium, were homogenized and lysed with TRIzol (Life Technologies, Carlsbad, CA, USA). The total RNA extraction, reverse transcription, and quantitative RT-PCR were performed as previously described.[49] Data were analyzed with the comparative cycle threshold method. Primers used were the following: TNF *sense* 5′-CATCTTCTCAAAATTCGAGTGACAA-3; TNF *anti-sense* 5′-TGGGAGTAGACAAGGTACAACCC-3′; GAPDH *sense* 5′-CATCTTCTTGTGCAGTGCCA-3′; GAPDH *anti-sense* 5′-CGGCCAAATCCGTTCAC-3′.

Western Blotting. RAW 264.7 macrophages were cultured as described earlier. After treatments, adherent cells were removed and the subcellular fractions were obtained following the protocol adapted from a previous report.[50] The cells were lysed with buffer A (10 mM HEPES, 10 mM KCl, 1 mM EDTA, 0.1 mM EGTA, 1 mM PMSF, 1 mM DDT, 0.2% NP40, protease inhibitor cocktail [Sigma] in ultrapure water). The homogenate was immediately transferred to tubes and vortexed for 1 min. The resulting extract was centrifuged at 20800*g* for 5 min, and the supernatant was collected as a cytoplasmic extract. The adhered pellet was washed twice with lysis buffer A, and the supernatants of the centrifugations from these washings were discarded. The resulting pellet was lysed with 50 μL of buffer B (20 mM HEPES; 420 mM NaCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM PMSF; 1 mM DDT; protease inhibitor cocktail in RIPA buffer [Sigma]) maintained in ice and homogenized by vortexing for 30 min. The resulting extract was centrifuged at 20800*g* for 5 min. The resulting supernatant was taken as a nuclear extract.

Samples of nuclear and cytoplasmic extracts had their protein content quantified by the Bradford method.[51] The proteins in each extract were separated by electrophoresis on 12% polyacrylamide gel (SDS-PAGE) followed by transfer to a nitrocellulose membrane. The membrane containing cytoplasmic proteins was incubated with rabbit anti-IκB-α (sc-371, Santa Cruz Biotechnology, Dallas, TX, USA) at a concentration of 1:1000 in filtered TBS-T buffer containing 5% milk powder overnight at 2−8 °C with stirring. The membrane containing the nuclear proteins was incubated with rabbit anti-p65 (sc-372, Santa Cruz Biotechnology), 1:300 concentration in filtered T-TBS buffer containing 5% milk powder. After incubation, the membranes were washed with TBS-T buffer and incubated again with a secondary antibody conjugated with peroxidase (anti-rabbit IgG, Sigma). For measurement, a chemiluminescence system (ECL Western Blotting Systems, GE Healthcare, Little Chalfont, BKM, UK) was used and visualized using the ChemiDoc XRS+ System (BioRad, Life Technologies). To assess the quality of the separation of the extracts, the same membranes were stained with the anti-mouse antibody nucleophosmin (Sigma) at a concentration of 1:1000 for the nuclear extract or mouse actin antibody (Sigma) 1:2500 for the cytoplasmic extract. Then, the membranes were incubated with respective secondary antibodies conjugated with peroxidase following the same protocol described above. The bands shown are representative of the groups. The quantification was performed by normalization with a control group (medium).

Immunofluorescence. RAW 264.7 cells were grown on coverslips inside a 12-well plate under the same conditions described previously. Cells were pretreated with vehicle, **1** (10 μM), or pyrrolidine dithiocarbamate (500 μM) 30 min prior to addition of the stimulus LPS (100 ng/mL) and then incubated at 37 °C under 5% CO_2 for 30 min. Supernatants were removed, and cells were fixed with 4% paraformaldehyde solution, pH 7.2, for 20 min. Cells on coverslips were washed between steps with 10 mM glycine in PBS or only PBS. Cells were permeabilized by 0.2% Triton solution in PBS for 30 min and then incubated for 1 h with the blocking solution containing 1% goat serum and 2% BSA. Primary antibody rabbit anti-p65 (sc-372, Santa Cruz) was used at a concentration of 1:100 in PBS. Secondary antibody, goat anti-rabbit conjugated with Alexa-Fluor 488 (A11034, Molecular Probes), was used at a concentration of 1:400. Hoechst 33342 dye (1 μg/mL) was used to label cell nuclei (Sigma). The coverslips were removed and fixed on lamina with Fluoromount-G (SouthernBiotech, Birmingham, AL, USA). Image acquisition was performed using a confocal Leica TCS SP5-AOBS microscope (Leica Microsystems, Mannheim, BW, Germany).

Transfection in RAW 264.7 Macrophages. RAW 264.7 cells maintained in supplemented RPMI-1640 medium were seeded in a 24-well plate at a concentration of 5×10^5 cells/well. Supernatants were replaced by RPMI-1640 supplemented with 2% fetal bovine serum, to which was added pmirGLO Dual-Luciferase plasmid (Promega), 1 μg/well, using FuGene 6 transfection reagent (Roche Life Science, Indianapolis, IN, USA) following the manufacturer's instructions. Cells were incubated for 6 h under 5% CO_2 at 37 °C, which is the optimal time after transfection (previously standardized). After this time, **1** (10−0.1 mM), dexamethasone (1 mM), pyrrolidine dithiocarbamate (500 μM), or cycloheximide (70 μM) was added, and cells were incubated for 6 h under 5% CO_2 at 37 °C. Cells were lysed with TNT buffer (0.1 M Tris·HCl (pH 7.5), 0.15 M NaCl, 0.05% Tween), and luminescence was determined as described above.

Statistical Analysis. The results are presented as mean ± SEM for the total number of representative experiments. Differences between the experimental groups were compared by one-way ANOVA. Where statistical significance was found, individual comparisons were subsequently made with Tukey's post hoc test. The level of significance was set at $p < 0.05$.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

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Notes

The authors declare no competing financial interest.

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