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Natural chromones as potential anti-inflammatory agents: Pharmacological properties and related mechanisms

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ABSTRACT

Chromones are a group of natural substances with a diversity of biological activities. Herein we assessed the pharmacological potential of three chromones (**1**, **2** and **3**) isolated from *Dictyoloma vandellianum* as anti-inflammatory agents using *in vitro* and *in vivo* approaches. During *in vitro* screening, the production of NO and cytokines by macrophages stimulated with LPS and IFN-γ was inhibited by all chromones at concentrations (5–20 μM) that did not induce cytotoxicity. Analysis of pharmacokinetic parameters (*in vitro* half-life and intrinsic clearance) using human liver microsomes revealed that **3** has a superior pharmacokinetic profile, compared to **1** and **2**. Treatment with **3** (100 mg/kg, ip) did not affect the mice motor performance, while **1** and **2** induced motor deficit. Taking into account the pharmacokinetic profile and absence of motor impairment, **3** was selected for further pharmacological characterization. Corroborating the data from *in vitro* screening, treatment of cell cultures with **3** (5–20 μM) reduced TNF-α, IL-6 and IL-1β production by stimulated macrophages. In the complete Freund's adjuvant-induced paw inflammation model in mice, **3** (25 and 50 mg/kg, ip) inhibited mechanical hyperalgesia, edema and cytokine production/release (IL-1β, IL-6 and TNF-α). **3** (5–20 μM) also reduced the transcriptional activity of NF-κB in stimulated macrophages. Furthermore, treatment with RU486, a glucocorticoid receptor (GR) antagonist, partially prevented the inhibitory effect of **3** on macrophages, indicating that this chromone exerts its anti-inflammatory effects in part through the activation of GR. The results presented herein demonstrate the pharmacological potential of natural chromones, highlighting **3** as a possible candidate for the drug discovery process targeting new anti-inflammatory drugs.

1. Introduction

Inflammation is a physiological response to harmful stimuli, aiming at eliminating the cause of the injury and promoting the healing process. Although it has an important physiologic function, a large body of evidence shows that chronic inflammation can be the primary cause or part of the stimuli that induces the establishment of a large number of diseases, such as asthma, rheumatoid arthritis, cancer, neuropathies and neurodegenerative diseases [1]. The inflammation underlying chronic diseases affects many patients in terms of physical limitations, persistent pain and diminished quality of life, causing a significant social and economic burden [2].

Reflecting the aging of the world population, which is associated with a higher prevalence of chronic diseases, the anti-inflammatory drug market grew in 2010 at a rate of 7.6% to $57.8 billion [3], and it is expected to garner $106.1 billion by 2020 [4]. Current pharmacological treatment of inflammation relies on the use of nonsteroidal anti-inflammatory drugs and glucocorticoids, which are essential drugs in controlling a wide range of diseases and conditions. However, their adverse effects may be significant, especially in higher doses and for use chronically or in elderly people [5,6], stressing the demand for new anti-inflammatory agents.

Historically, natural products have been, and are currently, a plentiful source of new chemical entities with anti-inflammatory properties. Chromones (4H-chomen-4-ones) are an important class of compounds with widespread occurrence in nature, particularly in

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plants. This benzopyrone moiety has been considered a privileged structure due to the variety of pharmacological properties of its derivatives, considered very interesting from a pharmacological and medicinal chemistry point of view [7,8]. It has been reported that molecules containing the chromone scaffold are bioactive, exhibiting pharmacological properties, such as anti-inflammatory, antiallergenic and antihypertensive actions [9,10].

Regarding their anti-inflammatory activity, chromones have been identified as cyclooxygenase inhibitors, leukotriene receptor antagonists, mast cell stabilizers, interleukin-5 inhibitors, intercellular adhesion molecule inhibitors and NO production inhibitors [9]. Importantly, chromone derivatives presenting anti-inflammatory activity are clinically used, such as nedocromil and cromoglycate, both used for asthma, and Iguratimod, used for treating rheumatoid arthritis as a disease-modifying agent, highlighting the potential of chromones for pharmaceutical development [11–13]. Therefore, the present study aims to evaluate the potential anti-inflammatory activity of chromones, 6-(3-methylbut-2-enyl) allopteroxylin methyl ether (**1**), 3,3-dimethylallylspatheliachromene methyl ether (**2**) and 5-*O*-methylcneorumchromone K (**3**), isolated from the root bark of *Dictyoloma vandellianum*, and to investigate their possible mechanisms of action.

2. Materials and methods

2.1. Animals

The present study was performed on male Swiss Webster mice (24–28 g) obtained from the Animal Facilities at Instituto Gonçalo Moniz (Salvador, Brazil). Animal care and handling procedures were in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of National Institute of Health and the Institutional Animal Care and Use Committee of FIOCRUZ (permit number: L-IGM-015/2013). Behavioral tests were performed as blinded-experiments.

2.2. Test compounds and stimuli

Chromones were isolated from the root bark of *Dictyoloma vandellianum* (Rutaceae) collected in March 2005 in Piatã/BA, Brazil (13°140 43″S, 41°450 28″W). The plant was identified by Dr. Maria *Lenise* Silva Guedes from the Herbarium Alexandre Leal Costa of the Federal University of Bahia, Brazil. A voucher specimen (n°. 69163) has been deposited at the Herbarium Alexandre Leal Costa. The procedures used for purification of chromones, 6-(3-methylbut-2-enyl) allopteroxylin methyl ether (**1**), 3,3-dimethylallylspatheliachromene methyl ether (**2**) and 5-*O*-methylcneorumchromone K (**3**), have been described [14]. The percent purity of chromones determined by HPLC was greater than 98%. The endotoxin content of the chromones solutions was determined using the LAL Chromogenic Endotoxin Quantitation Kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. The endotoxin content was ≤ 0.40 EU/mL for all tested compounds.

Dexamethasone, antagonist of glucocorticoid receptor RU486, complete Freund's adjuvant (CFA), lipopolysaccharide (LPS) and interferon-γ (IFN-γ) were obtained from Sigma Chemical Company (St. Louis, MO, USA). Diazepam was obtained from Cristália (Itapira, SP, Brazil). Dexamethasone was dissolved in ethanol (10% in normal saline), while chromones were dissolved in 50% propylene glycol plus saline. Remaining substances were dissolved in saline. Treatments were performed 40 min before testing, by intraperitoneal (ip) route.

2.3. Cytotoxicity to mammalian cells

To determine the chromones cytotoxicity, murine macrophage-like cells line J774 were plated into 96-well plates, as previously described [15]. Chromones were added to the plates at five concentrations ranging from 3.125 to 50 μM in triplicate and incubated for 72 h. Alamar Blue (20 μL/well; Invitrogen, Carlsbad, CA, USA) was added and 12 h later the plate colorimetric measurements (570 and 600 nm) were performed. The positive control was gentian violet (Synth, São Paulo, Brazil) at 10 μM.

2.4. Cytokine and nitric oxide production by macrophages

The anti-inflammatory potential of chromones was first evaluated in J774 cells stimulated with LPS (500 ng/mL, Sigma) and IFN-γ (5 ng/mL; Sigma) as previously described [15]. Cells were stimulated with LPS + IFN-γ in the presence of chromones, vehicle or dexamethasone at different concentrations, and incubated at 37 °C. Cell-free supernatants were collected 4 h (for TNF-α measurement) and 24 h (for IL-10, IL-6 and nitrite quantification) and kept at −80 °C. Cytokine concentrations were determined by enzyme-linked immunosorbent assay (ELISA), using the DuoSet kit from R&D Systems (Minneapolis, MN, USA), according to the manufacturer's instructions. Quantification of nitrite as an indicator of nitric oxide production was done using the Griess method [16]. For the antagonism assay, the glucocorticoid receptor antagonist RU486 (Sigma) was added in some cultures at a final concentration of 10 μM.

2.5. Apoptosis quantification assay

For apoptosis quantification, Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit I (BD Biosciences, San Jose, CA, USA) was used, according to the manufacturer's instructions. Briefly, J774 macrophages (5×10^5 cells/well) were plated in supplemented DMEM medium, followed by incubation with chromones (20 μM) for 24 h at 37 °C with 5% CO_2. Then, the cells were trypsinized and the apoptosis quantification assay was performed. Cell fluorescence was measured by flow cytometry with a FACSCalibur flow cytometer (BD). For flow cytometry analyses, 10,000 events were recorded per sample, using the Flowjo Software 7.5 (Flowjo LLC, Ashland, OR, USA).

2.6. Incubation with liver microsomes

Pooled human male liver microsomes (HLM) were purchased from Sigma-Aldrich (Steinheim, Germany). These microsomes were characterized in protein and CYP content. All incubations contained chromones (100 μM) as substrate, an NADPH-regenerating system (5 mM glucose-6-phosphate, 1 mM $NADP^+$ and 5 units/mL glucose-6-phosphate dehydrogenase) and 4 mM $MgCl_2{\cdot}6H_2O$ in 0.1 M Tris buffer (pH 7.4 at 37 °C) and were pre-incubated for 5 min at 37 °C and 750 rpm. The incubation mix was split into 100 μL aliquots and the reactions were started by addition of the HLM. Thereby the microsomal protein content was standardized to 1 mg/mL. To follow the course of metabolic degradation, reaction tubes were quenched at selected time points (0, 10, 20, 30, 60, 120, 180 and 240 min) by adding 400 μL acetonitrile containing the internal standard. Samples were vortexed and centrifuged at 13,500 rpm and 4 °C for 20 min to pellet precipitated proteins. An aliquot of each supernatant was used for LC-MS analysis. All incubations were conducted in triplicates and incubations with heat-inactivated HLM were used to proof that analyte reduction results from metabolic degradation only, as described previously [17].

2.7. Metabolic stability and pharmacokinetic parameters determination

Substrate degradation was analyzed with an Alliance 2695 Separations Module (Waters GmbH, Eschborn). Samples were maintained at 4 °C, the column temperature was set to 40 °C and injection volume was 10 μL. The chromatographic separation was performed on an EC 150/3 Nucleodur Sphinx RP; 3 μm (150.0 × 3.0 mm; Macherey Nagel™) column, preceded by a precolumn of the same material. A binary gradient consisting of solvent A (0.1% formic acid in water:

acetonitrile 90:10, v/v) and solvent B (0.1% formic acid in acetonitrile) for 10 min at a flow rate of 400 μL/min was used for separation by high performance liquid chromatography. The initial composition of 10% B was held for 2 min, followed by a linear gradient up to 90% B in 5 min, immediately changing to 10% B and reequilibration at the end. The detection was performed on a Micromass Quattro micro triple quadrupole mass spectrometer (Waters GmbH, Eschborn) using the electrospray ionization in the positive-mode. Spray voltage was set to 3.0 kV. The heated capillary operated at 250 °C and the desolvation gas flow worked at 500 L/h. Peak area ratios of chromones against the internal standard were used to determine the amount of parent remaining compared to that at 0 min. The percent of chromones remaining was calculated by dividing the peak area ratio obtained at each time point by that obtained at 0 min. *In vitro* $t_{1/2}$ was calculated as described previously [18] and *in vitro* intrinsic clearance (CL_{int}) was calculated as described by Obach [19]. Three independent experiments (n = 3) were performed for each compound.

2.8. Motor function test

A possible interference of chromones in the motor function of the mice was evaluated in the rota-rod (Insight, Ribeirão Preto, SP, Brazil) test, as previously described [20]. Mice received intraperitoneal administration of chromones (100 mg/kg) or diazepam (10 mg/kg), used as positive control, and 40 min afterwards were placed on a rotating rod (6 rpm).

The permanence time of the mice on the rota-rod was recorded for up to 120 s.

2.9. Inflammatory model

The complete Freund's adjuvant (CFA)-induced paw inflammation model in mice was performed here according to a previously reported method [15,21], and the inflammatory parameters evaluated included: hyperalgesia, edema, and local cytokine levels. Mice were injected with **3** (50, 25, 12.5 or 6.25 mg/kg), vehicle (50% propylene glycol in saline; control group), or dexamethasone (2 mg/kg, reference drug) by ip route 40 min before CFA. Mice were anesthetized with halothane and received CFA (20 μL, 1 mg/mL of heat-killed *Mycobacterium tuberculosis* in 85% paraffin oil and 15% mannide monoleate; Sigma) subcutaneously in the hind paw. The mechanical nociceptive threshold was measured with von Frey filaments (0.008–2.0 g; Stoelting, Chicago, IL, USA), using the up − down method adapted by Chaplan and coworkers [22]. The nociceptive threshold was represented as the filament weight (g) in which the animal responds in 50% of presentations. The volume of mouse paw was measured with a plesthismometer (Ugo Basile, Comerio, Italy) before and after the CFA injection, and data were represented as paw volume variation (Δ, mm^3).

2.10. Cytokine measurement by ELISA

Cytokine levels in the inflamed paw of mice were determined by ELISA, as previously described [15]. Mice were injected with **3** (50 mg/kg), vehicle (5% DMSO in saline; control group), or dexamethasone (2 mg/kg, reference drug) by ip route 40 min before the CFA injection. Skin tissues were removed from the paws 2, 4 and 24 h after CFA injections in mice terminally anesthetized. The naïve group consists of mice that did not receive any experimental manipulation. Tissue processing and ELISA procedures were performed according to the ELISA kits (R&D System) manufacturer's instructions and as previously described [23]. Interleukin 1β (IL-1β), Interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α) levels were expressed as picograms of cytokine per milligram of protein.

2.11. Nuclear transcription factor-κB (NF-κB) luciferase assay

The transcriptional activity of NF-κB in macrophages was investigated according to a previously reported method [21]. Macrophage cell line Raw 264.7 Luc bearing the pBIIX-luciferase (pBIIX-luc) targeting vector containing the firefly luciferase gene (luc) driven by two NF-kB binding sites from the kappa light chain enhancer in front of a minimal *fos* promoter [24] were pretreated with different concentrations of **3** (20, 10 or 5 μM) for 1 h and then stimulated with LPS (500 ng/mL) and IFN-γ (5 ng/mL) for 3 h. Each well was washed and cells were incubated with TNT lysis buffer for 20 min at 4 °C. The luciferase activity in cell lysates was determined using the Luciferase Assay System (Promega, Madison, WI), in Globomax 20/20 luminometer (Promega). Data were expressed as relative light units.

2.12. Statistical analysis

Data are presented as mean ± standard deviation (SD), with a minimum sample of n = 6 per group. Comparisons between groups were made using one-way ANOVA with Tukey post-hoc test or two-way ANOVA with Bonferroni post-hoc test for repeated measures data. Analyzes were performed using Prism 5 Computer Software (GraphPad, San Diego, CA, USA), with statistical significance at $p < 0.05$.

3. Results and discussion

The anti-inflammatory potential of the natural chromones **1**, **2** and **3** was demonstrated for the first time in the present investigation. The *in vitro* tests showed that chromones exhibited minimal cytotoxicity and suppressive effects on activated macrophage. A further evaluation of the *in vivo* pharmacological properties of **3** showed that this chromone attenuated the paw edema and inflammatory hyperalgesia and inhibited the local increase of key cytokines related to inflammation, namely IL-1β, TNF-α and IL-6, possibly through the activation of glucocorticoid receptors and inhibition of NF-κB transcriptional activity.

Initially, the macrophage stimulation assay was used as an *in vitro* screening aiming to determine the anti-inflammatory potential of chromones. Macrophages take part in immune regulation and inflammatory processes as a key cell, acting through antigen presentation, phagocytosis and immunomodulation [25]. Since activated macrophages are important producers of inflammatory mediators, the effect of chromones on the production of nitric oxide and cytokines by stimulated macrophages was evaluated. To avoid misinterpretation, the effect of chromones on cell viability in stimulated and unstimulated J774 macrophages was first evaluated. As shown in Fig. 1A, C and E, chromones did not induce cytotoxic effects in stimulated J774 macrophages at concentrations under 25 μM. The cytotoxic profile of chromones in unstimulated J774 macrophages was similar to that observed in stimulated cells (data not shown in the figure). Analysis of annexin V FITC corroborated the cytotoxicity data. The percentages of annexin V positive cells were: 3.9 ± 1.9% for the blank control group; 3.5 ± 1.6% for the vehicle group; 2.8 ± 1.1% for **1** at 20 μM; 3.4 ± 0.9% for **2** at 20 μM; 4.6 ± 1.9% for **3** at 20 μM. Analysis of these data showed that the percentage of cells in apoptotic stage after 24 h of incubation with chromones (20 μM) was similar to that observed in the blank control or vehicle groups. Based on these studies, subsequent experiments were performed within the non-cytotoxic concentration range. **1**, **2** and **3** (5, 10 and 20 μM) were able to reduce the amount of nitrite on macrophages stimulated with LPS and IFN-γ in comparison to vehicle treated stimulated cells ($p < 0.001$; Fig. 1B, D and F, respectively), suggesting a reduction of nitric oxide production. Furthermore, **1**, **2** and **3** at 20 μM reduced the levels of the cytokines TNF-α (Fig. 1G; $p < 0.01$) and IL-6 (Fig. 1H; $p < 0.05$). Incubation of unstimulated J774 macrophages cultures with **1**, **2** and **3** at 20 μM for 4 and 24 h did not induce the production of nitrite and cytokines by these cells. In all *in vitro* assays, no statistical differences were observed

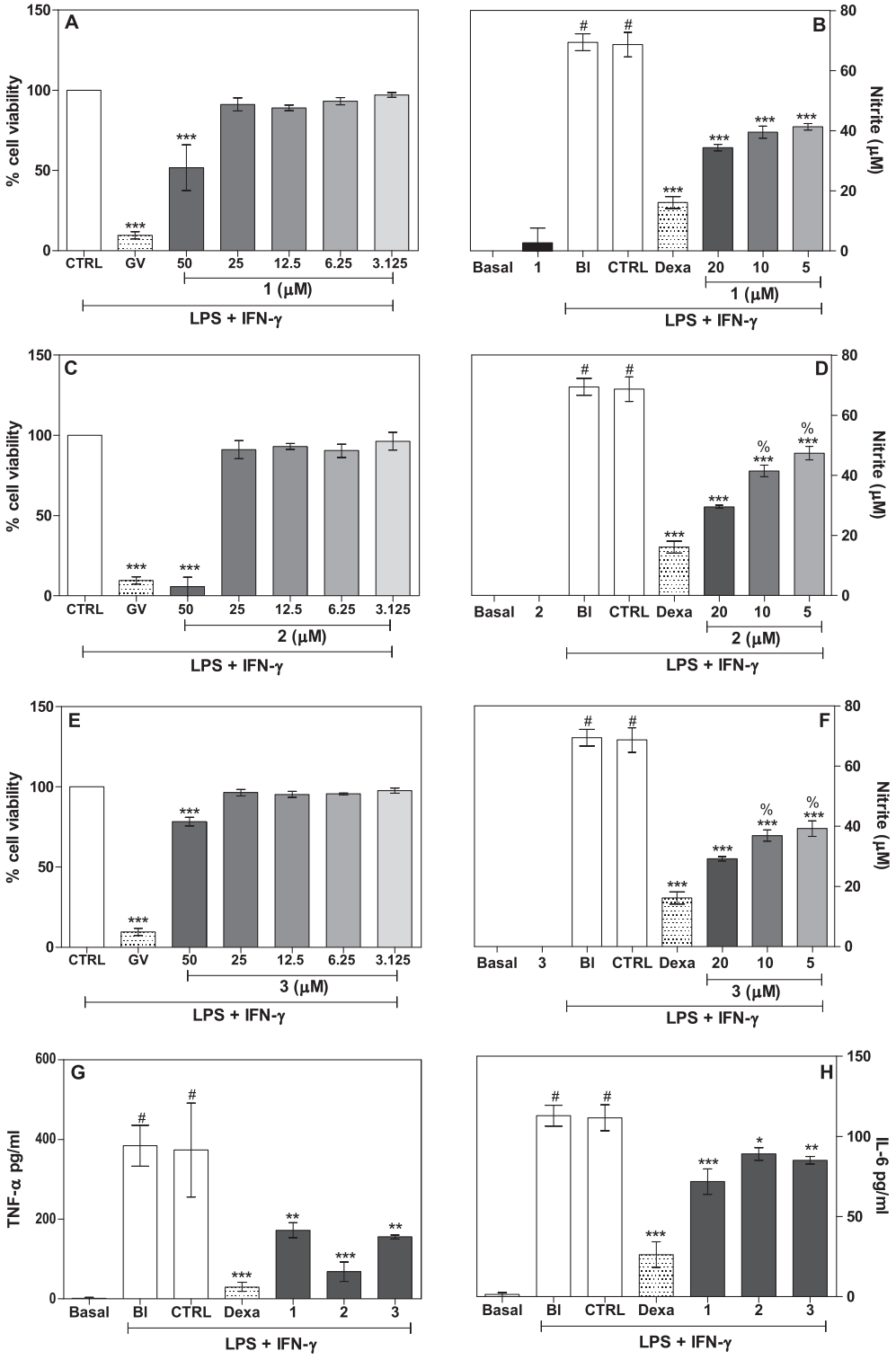

Fig. 1. Chromones induce low cytotoxicity and inhibitory effect on the production of nitric oxide and cytokines by stimulated J774 macrophages. Panels A, C and E show cell viability data determined by the Alamar Blue assay. Vehicle (50% propylene glycol in saline, CTRL), gentian violet (GV; positive control) or different concentrations of 1 (A), 2 (C) or 3 (E) were added to the macrophages cultures and incubated for 72 h. ***Different from the vehicle group ($p < 0.001$). Panels B, D and F show nitrite quantification determined by the Griess method. Vehicle (50% propylene glycol in saline, CTRL), dexamethasone (Dexa; 40 μM, reference drug) or different concentrations of **1** (B), **2** (D) or **3** (F) were added to J774 macrophages cultures in the presence of LPS (500 ng/mL) + IFN-γ (5 ng/mL). Nitrite quantifications were performed 24 h after treatments. Blank control group (Bl) represents untreated cells stimulated with LPS + IFN-γ. Basal group shows data from untreated and unstimulated cells. Groups 1, 2 and 3 show data from unstimulated cells treated with 1, 2 and 3 at 20 μM. #Different from the unstimulated groups ($p < 0.001$). ***Different from the vehicle and blank control groups ($p < 0.001$). %Different from the 20 μM group ($p < 0.05$). Panels G and H show cytokines quantification data by ELISA. Vehicle (50% propylene glycol in saline, CTRL), chromones (1, 2 and 3; 20 μM) or dexamethasone (Dexa; 40 μM) were added to macrophages cultures stimulated with LPS + IFN-γ. Blank control group (Bl) represents untreated cells stimulated with LPS + IFN-γ. TNF-α (G) and IL-6 (H) levels were determined, respectively, 4 h and 24 h after treatments. Basal group shows data from unstimulated cells. #Different from the basal group ($p < 0.001$). *Different from the vehicle and blank control groups ($p < 0.05$). **Different from the vehicle and blank control groups ($p < 0.01$). ***Different from the vehicle and blank control groups ($p < 0.001$). Data are expressed as mean ± SD; n = 6. ANOVA followed by Tukey's test.

between the blank control and vehicle groups, indicating that the vehicle used does not interfere with the biological responses in these assays. As expected, dexamethasone, used as reference drug, was able to inhibit NO and cytokines release by stimulated macrophages ($p < 0.001$). Several compounds containing the chromone moiety were able to inhibit iNOS expression and NO production, which is one of the mechanisms related to their anti-inflammatory activity [26–29]. These results suggest that **1**, **2** and **3** present anti-inflammatory potential due to the important role of cytokines and NO as pro-inflammatory agents [30,31].

Metabolic stability determination *in vitro* has served as a tool for selecting new chemical entities with the most suitable properties for drug development [19,32], and therefore was used in the present study to characterize the chromones potential for drug development. *In vitro* half-life and intrinsic clearance (Cl_{int}) of chromones were evaluated to provide an indication of the metabolic stability and preclinical pharmacokinetic parameters of these substances. **1** and **2** exhibit a very similar profile, whereas **3** seems to be more metabolically stable. Data presented in Table 1 displays the *in vitro* half-life and intrinsic clearance of **1**, **2** and **3** and shows that **3** has higher metabolic stability (86.61%)

Table 1
Pharmacokinetic parameters of chromones.

Substance	$t_{1/2}$ (hours)[a]	Cl_{int} (mL/min/kg)[b]	Met (%)[c]
1	3.13 ± 0.44	3.36 ± 0.45	54.05 ± 3.04
2	2.04 ± 0.46	5.22 ± 0.95	56.14 ± 1.28
3	22.61 ± 2.59	0.57 ± 0.27	89.61 ± 0.62

[a] $t_{1/2}$ - *in vitro* half-life.
[b] Cl_{int} - intrinsic clearance.
[c] Percent remaining after 4 h of incubation with HLM.

and half-life (25.02 ± 4.08 h) than **1** and **2** (54.05%, 56.14%, 4.40 ± 0.22 h and 4.88 ± 0.15 h, respectively). In addition, under experimental conditions, and in the absence of variables such as hepatic blood flow and plasma protein binding, **3** has lower liver clearance, as evidenced by the lower Cl_{int} for **3** (0.42 ± 0.05 mL/min/kg) compared to **1** (2.37 ± 0.14 mL/min/kg) and **2** (2.13 ± 0.05 mL/min/kg). It is important for drug candidates to have a good metabolic stability, since this feature is associated with an increased bioavailability, longer half-life and better relation between dose and plasma concentration [33]. Moreover, a lower hepatic clearance is related to a lower individual metabolic variability, as hepatic clearance is replaced by renal clearance [32]. Therefore, the present data from metabolic stability and pharmacokinetic profiling *in vitro* studies suggest that **3** presents an improved metabolic profile relative to **1** and **2**.

Before conducting specific pharmacological tests *in vivo*, possible systemic side effects of chromones, such as motor impairment, sedation and ataxia, were investigated by using the rota-rod test (Fig. 2). Intraperitoneal administration of **3** (100 mg/kg) did not alter the mice performance on the test, while **1** and **2** at 100 mg/kg reduced the running time of mice on the rota-rod apparatus ($p < 0.05$ and $p < 0.001$, respectively). The running time of mice treated by intraperitoneal route with diazepam (10 mg/kg), used herein as a positive control, was also reduced ($p < 0.001$). These results may reflect a better profile of systemic side effects for **3** compared to **1** and **2**. Taking in account the *in vitro* pharmacokinetic parameters and the results of the rota-rod test, **3** was selected for further pharmacological investigation.

In order to better characterize the inhibitory effect of **3** on inflammatory mediators released by macrophages, concentration-response curves of **3** were performed. In line with the preliminary results (Fig. 1G and H), **3** reduced the production of pro-inflammatory

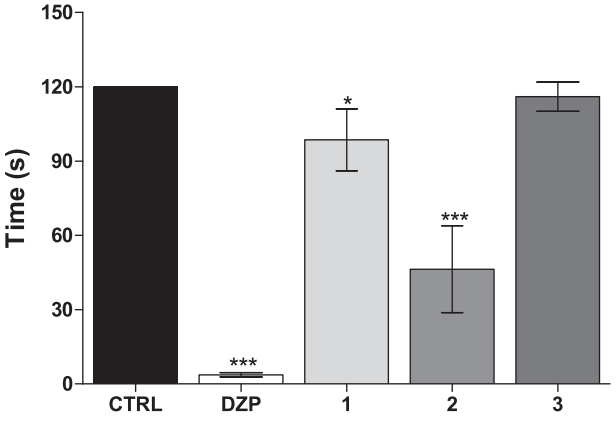

Fig. 2. Effects of chromones on mice performance on the rota-rod test. Mice were treated intraperitoneally with **1**, **2** or **3** (100 mg/kg), vehicle (CTRL, 50% propylene glycol in saline) or diazepam (DZP; 10 mg/kg, positive control) and, 40 min later, the run time on the rota-rod was evaluated. Data are expressed as mean ± SD; n = 6. *Different from the vehicle group ($p < 0.05$); ***Different from the vehicle group ($p < 0.001$), as determined by one-way ANOVA followed by Tukey's test.

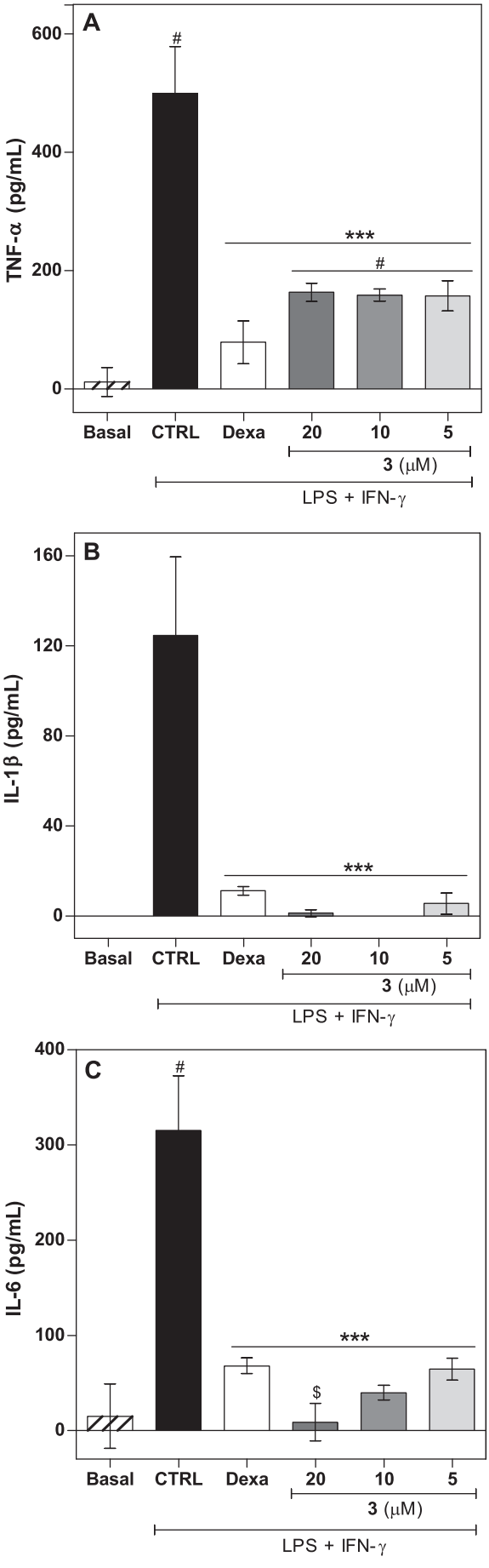

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Fig. 3. Production of pro-inflammatory cytokines by stimulated macrophages is inhibited by 3. Figure shows cytokines quantification data in J774 macrophages cultures. Vehicle (50% propylene glycol in saline, CTRL), **3** (20, 10 or 5 μM) or dexamethasone (Dexa; 40 μM) were added to macrophages cultures stimulated with LPS (500 ng/mL) + IFN-γ (5 ng/mL). TNF-α (A), IL-1-β (B) and IL-6 (C) levels were determined by ELISA 4 h and 24 h after treatment, respectively. Basal group shows data from unstimulated cells. ***Different from the vehicle group ($p < 0.001$). #Different from the basal group ($p < 0.001$). $Different from the dexamethasone group ($p < 0.05$). Data are expressed as mean ± SD; n = 6. ANOVA followed by Tukey's test.

cytokines by macrophages (Fig. 3). Treatment with **3** at 20, 10 and 5 μM reduced the production of TNF-α (panel A), IL-1β (panel B) and IL-6 (panel C) by macrophages stimulated with LPS and IFN-γ ($p < 0.001$). This inhibitory effect was not concentration-dependent. Dexamethasone (40 μM), the reference drug, induced a reduction in TNF-α and IL-1β levels of a magnitude similar to **3**. On the other hand, the inhibitory effect of **3** on IL-6 levels was greater ($p < 0.05$) than that obtained with dexamethasone, considered a gold standard in this assay. In line with the present results, a series of chromones derivatives was found to have inhibitory activity on inflammatory cytokines (TNF-α and IL-6) [34]. Nedocromil and cromoglycate, clinically used chromones derivatives, also inhibited the production/release of pro-inflammatory cytokines [35,36].

Next, the anti-inflammatory properties of **3** *in vivo* were investigated using the CFA-induced paw inflammation test, which is a robust model of persistent inflammation. CFA induces a local inflammatory process characterized

by redness, hyperalgesia and edema, associated with the production of pro-inflammatory mediators, such as prostanoids and cytokines [37–39]. Thus, using CFA as inflammatory stimulus, parameters such as mechanical hyperalgesia, edema and cytokine production were used to characterize the anti-inflammatory properties of **3**. Mice were pretreated with **3** (50–6.25 mg/kg, ip), vehicle or dexamethasone (2 mg/kg; reference drug) 40 min before the CFA injection, then mechanical hyperalgesia and paw edema were measured within the following 48 h (Fig. 4). In vehicle-treated mice, the mechanical nociceptive threshold was strongly reduced 2 h after CFA injection, characterizing the development of mechanical hyperalgesia due to local inflammation, which persisted throughout the experimental period (Fig. 4A). Intraperitoneal administration of **3** at 50 mg/kg reduced mechanical hyperalgesia until 24 h after CFA ($p < 0.05$), while the antinociceptive effect of **3** at 25 mg/kg persisted 8 h ($p < 0.05$). **3** at 12.5 or 6.25 mg/kg, as well as vehicle, did not change the nociception. Dexamethasone (2 mg/kg) induced antinociceptive effect until 24 h after the stimuli ($p < 0.05$).

Following CFA injection, vehicle-treated mice developed a gradually increasing paw edema (Fig. 4B) that peaked after 24 h. Pretreatment with **3** (50–6.25 mg/kg), but not with vehicle, greatly reduced paw edema, and this antiedematogenic effect persisted for 24 h ($p < 0.05$). As expected, dexamethasone also reduced the paw edema ($p < 0.05$). The anti-inflammatory effects of **3** *in vivo* were comparable to dexamethasone, the gold standard, both in efficacy and in the long-lasting profile, highlighting the pharmacological potential of this chromone. Corroborating this idea, the anti-inflammatory properties of several chromones have been described. A C-glucosyl chromone found in *Aloe vera* showed antiedematogenic property in ear edema test [40]. A series of synthesized phenoxychromones showed significant anti-inflammatory activity in the carrageenan-induced edema and CFA-induced arthritis tests [41].

In order to validate the *in vitro* data, and achieve a further characterization of the anti-inflammatory profile of **3**, the modulatory effects of this chromone on the cytokine production were investigated *in vivo* (Fig. 5). CFA induced a strong increase in levels of TNF-α, IL-1β

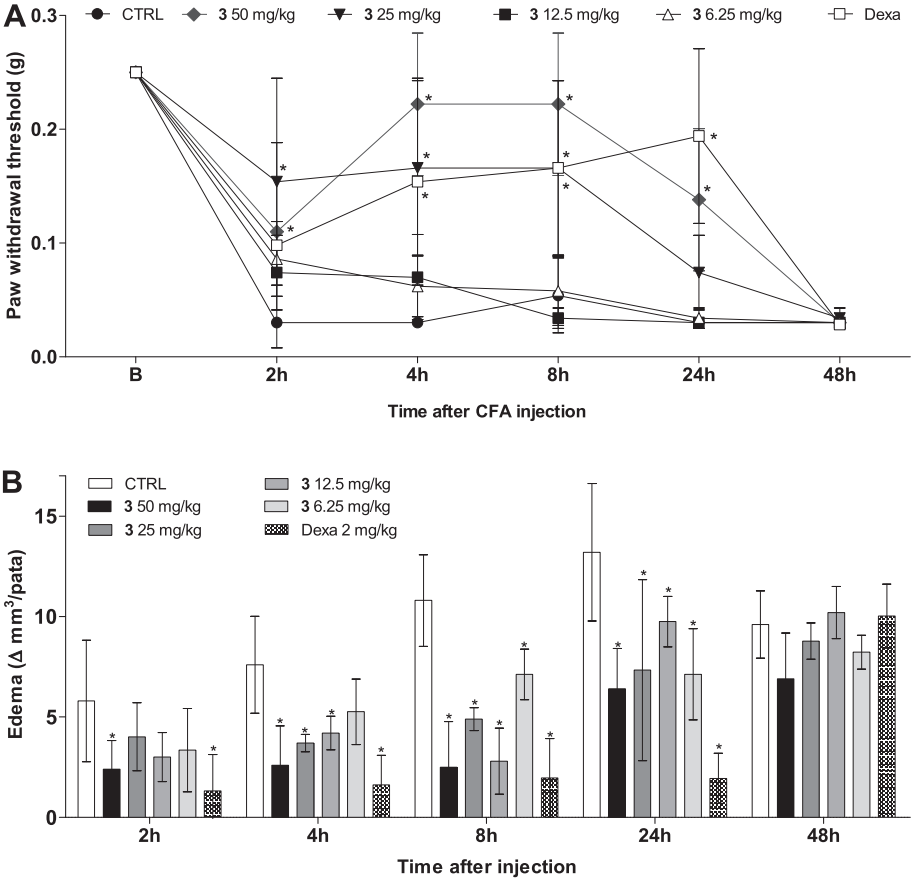

Fig. 4. Anti-inflammatory effects of 3 on CFA-induced paw inflammation. Panels A and B show, respectively, mechanical hyperalgesia and paw edema at different time points after CFA. Forty minutes prior to CFA, mice were intraperitoneally treated with **3** (6.25–50 mg/kg), vehicle (50% propylene glycol in saline; CTRL), or dexamethasone, used as reference drug (Dexa; 2 mg/kg). The paw withdrawal threshold (panel A) corresponds to the weight of the filament (g) in which the animal responds in 50% of the presentations. Edema (panel B) was represented as paw volume variation. Data are expressed as mean ± SD; n = 6. *Different from the vehicle group ($p < 0.05$). Two-way ANOVA followed by Bonferroni's test.

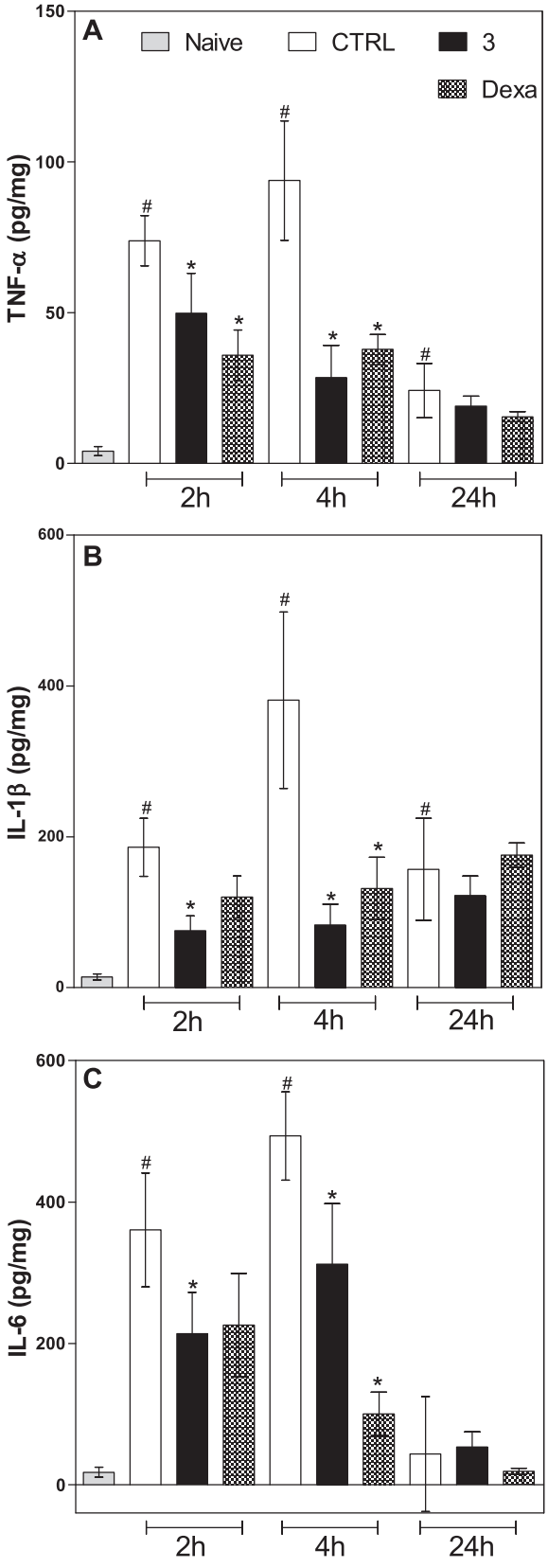

Fig. 5. Inhibitory effect of 3 on the local production of pro-inflammatory cytokines during CFA-induced inflammation in mice. Panels A, B and C show, respectively, the paw levels of TNF-α, IL-1β and IL-6 at different time points after CFA. Forty minutes prior to CFA injection, mice were intraperitoneally treated with **3** (50 mg/kg), vehicle (50% propylene glycol in saline; CTRL), or dexamethasone, used as reference drug (Dexa; 2 mg/kg). Naive mice were not experimentally manipulated. Data were plotted as picograms of cytokine per milligram of total protein, mean ± SD, n = 6. *Different from the vehicle group in the same time ($p < 0.05$); #Different from the naive group ($p < 0.05$). ANOVA followed by Tukey's test.

and IL-6 in inflamed paw tissue ($p < 0.05$). **3** (50 mg/kg) inhibited the local production of TNF-α (panel 5A), IL-1β (panel 5B) and IL-6 (panel 5C) 2 and 4 h after CFA-induced paw inflammation ($p < 0.05$). Dexamethasone (2 mg/kg) reduced the paw levels of TNF-α 2 and 4 h after CFA ($p < 0.05$), while IL-1β and IL-6 levels were reduced by this treatment only at the time point of 4 h.

It is widely accepted that cytokines contribute to the development of signs and symptoms of inflammation. Not only inflammatory cells but also other cell types, such as neurons and glial cells, produce IL-1β, TNF-α and IL-6 [31], which amplify the inflammatory response by inducing the production of other mediators involved in a wide range of inflammatory events [42–47]. TNF-α, IL-1β and IL-6 are considered essential mediators for the development of inflammatory hyperalgesia [39,48,49]. Importantly, the cytokine responses have been shown to be involved in the initiation, evolution and ultimately the resolution of some forms of inflammatory diseases in human [50–53]. In line with this idea, inhibitors of cytokine production have been considered a superior therapeutic strategy for inflammatory diseases, considering that they may exhibit disease-modifying profile [54–56]. In fact, recently a chromone, Iguratimod, has emerged as a new option of a disease modifying antirheumatic agent [13,57]. Iguratimod inhibits IL-1, IL-6, IL-8 and TNF-α production and on the molecular level, the activity of NF-κB [13,58,59], one of the major inducible transcription factors that regulate cytokine synthesis during inflammatory response.

A large number of stimuli, including activation of Toll-like receptors in macrophages [25], leads to activation of NF-κB signaling pathway, and the genes transcriptionally regulated by NF-κB play an important role in immune and stress responses [60,61]. To better understand the anti-inflammatory mechanisms of **3**, a possible modulatory effect of this chromone on NF-κB was next investigated. For this, a NF-κB reporter system in Raw 264.7 cells transfected with p-NF-κB-Luc reporter plasmid was used. The results of this assay, expressed as mean relative light units, were: basal group 388 RLU; vehicle group 4713 RLU; dexamethasone group 2951 RLU; **3** at 20 μM group 1485 RLU; **3** at 10 μM group 1686 RLU; **3** at 5 μM group 1833 RLU. Analysis of these data showed that stimulation with LPS and IFN-γ markedly increased NF-κB-dependent transcriptional activity in relation to unstimulated cultures (Fig. 6A; $p < 0.001$), while stimulated macrophages treated with **3** (5, 10 and 20 μM) showed a marked reduction of NF-κB activity, relative to vehicle-treated stimulated cells ($p < 0.001$). The inhibitory effect of **3** on the NF-κB transcriptional activity, at all tested concentrations, was greater ($p < 0.001$) than that caused by dexamethasone (40 μM; $p < 0.001$).

Considering that many inflammatory mediators are regulated by NF-κB, it is possible that the reduction of the activity of this transcription factor is one of the mechanisms by which **3** induces anti-inflammatory effects. In line with this hypothesis, a great number of drugs used on the treatment of inflammatory diseases modulate NF-κB [61]. For example, corticosteroids, which represent one of the most commonly used classes of anti-inflammatory drugs, have some of their effects mediated by the reduction of NF-κB activity [61,62]. Based on the relevant interaction between NF-κB and the glucocorticoid receptor (GR), the involvement of GR in the pharmacological effects of **3** was then investigated using a pharmacological antagonism assay (Fig. 6B). Vehicle-treated stimulated macrophages exhibited high levels of TNF-α ($p < 0.001$), whereas stimulated macrophages treated with **3** (20 μM; $p < 0.001$) or dexamethasone (40 μM; $p < 0.001$) showed reduced levels of TNF-α. RU486 (10 μM), a GR antagonist, partially prevented the inhibitory effect of **3** (20 μM; $p < 0.05$) on TNF-α production by

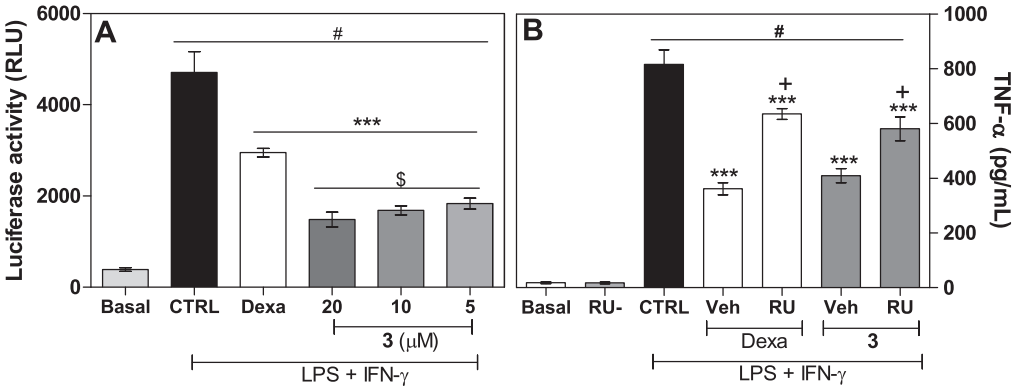

Fig. 6. Modulation of NF-κB transcriptional activity and glucocorticoid receptors contribute to the anti-inflammatory properties of 3. The panel A shows the effects of treatments on the transcriptional activity of NF-κB on RAW 264.7 Luc macrophages. Vehicle (50% propylene glycol in saline, CTRL), **3** (5, 10 or 20 μM) or dexamethasone (Dexa; 40 μM) were added to the macrophage cultures. One hour later, cultures were stimulated with LPS (500 ng/mL) and IFN-γ (5 ng/mL) for 3 h. Luciferase activity was measured in a luminometer as Relative Light Units (RLU). Basal group shows data from unstimulated cells. ***Different from the vehicle group ($p < 0.001$). #Different from the basal group ($p < 0.001$). $Different from the dexamethasone group ($p < 0.001$). Data from the glucocorticoid receptor antagonism assay are shown in panel B. The production of TNF-α by stimulated macrophages was the parameter selected to indicate pharmacological activity. Vehicle (50% propylene glycol in saline, CTRL), **3** (20 μM), RU486 (GR antagonist, 10 μM) + **3** (20 μM), dexamethasone (Dexa; 40 μM) or RU486 10 μM + dexamethasone 40 μM were added to macrophages cultures stimulated with LPS and IFN-γ. TNF-α levels were determined 4 h after treatments. Basal and RU-groups show data from unstimulated cells. Data represent the mean ± SD; n = 6. #Different from the basal group ($p < 0.001$). ***Different from the CTRL group ($p < 0.001$). +Different from the group not treated with antagonist ($p < 0.05$). ANOVA followed by Tukey's test.

stimulated macrophages (Fig. 6B). As expected, the inhibitory effect of dexamethasone (40 μM) was antagonized by RU486 ($p < 0.05$). The present data indicate that glucocorticoid receptor activation is likely one of the ways by which **3** exerts its anti-inflammatory effects. Corroborating the present findings, other compounds containing the benzopyrone moiety present interactions with GR, such as braylin [21], iguratimod [59] and icariin [63].

In conclusion, the present study represents the first demonstration of the *in vivo* pharmacological properties of chromones **1**, **2** and **3**. **3** exhibited relevant antinociceptive and anti-inflammatory effects, probably due to GR activation and inhibition of the NF-κB pathway. By demonstrating its improved pharmacological properties and favorable *in vitro* pharmacokinetic characteristics, the present study evidenced the potential of **3** for the drug discovery process, targeting new anti-inflammatory drugs with disease modifying profile.

Author contributions statement

Study concept and design: EAL and CFV. Acquisition of data: LCFO, RFES, OAN, LSA and IMA. Analysis and interpretation of data: LCFO, RFES, ED and CFV. Drafting and critical revision of the manuscript: LCFO, ED, MBPS, ESV, EAL and CFV. All authors read and approved the final manuscript.

Conflict of interest

The authors declare no competing financial interest.

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