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Short communication

Synthesis and inhibitory activity of dimethylamino-chalcone derivatives on the induction of nitric oxide synthase

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Abstract

A series of nine dimethylamino-chalcone derivatives (1,3-diaryl-propenones) was synthesized and screened as potential inhibitors of NO and PGE_2 production in the RAW 264.7 macrophage cell line. 4-Dimethylamino-2′,5′-dimethoxychalcone (**6**) was found to be the most potent and dual inhibitor (IC_{50s} in the submicromolar range) of NO and PGE_2 production. 2′,6′-Dimethoxylation appeared to be an effective requirement for selective and potent inhibition of nitric oxide synthase induction as it was confirmed by Western blot analysis. Chalcone (**6**) at 25 mg kg^{-1} by oral route, inhibited significantly the formation of oedema in the carrageenan-induced model of inflammation in mice. © 2002 Éditions scientifiques et médicales Elsevier SAS. All rights reserved.

Keywords: Dimethylamino-chalcones; Synthesis; Nitric oxide; Cyclooxygenase-2; RAW 264.7

1. Introduction

Macrophages constitutively produce prostaglandins by means of the enzyme cyclooxygenase-1, whereas inflammatory mediators and cytokines stimulate the inducible enzymes cyclooxygenase-2 and inducible NO synthase thus promoting high output of prostaglandin and NO [1,2]. Reactive nitrogen intermediates such as NO play an important role in inflammatory and immune reactions. Therefore, in addition to acting as a powerful effector molecule mediating the cytotoxic activities of mouse macrophages, NO can play a role in enhancing the production of a variety of other inflammatory mediators and can contribute both directly and indirectly to the immunopathology of macrophage-dependent inflammation [3]. Inhibition of the production of NO and prostaglandin E_2, by affecting the expression of those inducible enzymes involved in their production [4] could be an important strategy to obtain promising anti-inflammatory agents. A great number of chalcone derivatives have been reported as anti-inflammatory agents [5–7]. In this regard, we have described previously the inhibitory effect of 2′-hydroxy-3′,4′,3,4-tetramethoxychalcone on the generation of eicosanoids [8] and some other chalcone derivatives [9–11]. The present study was undertaken to synthesize and evaluate the activity of nine dimethylamino-chalcone derivatives, also named 1,3-diaryl-propenones (Table 1), against NO and PGE_2 production on the mouse macrophage cell line RAW 264.7. Chalcones **1** and **7** have been described chemically [12,13].

2. Chemistry

The general synthetic strategy employed to prepare the dimethylamino-chalcone derivatives was based on Claisen–Schmidt condensation, which has been previously reported [14]. As shown in Table 1, a series of nine dimethylamino-chalcones derivatives (**1**–**9**) were

Abbreviations: COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; IC_{50}, inhibitory concentration 50%; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NO, nitric oxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PGE_2, prostaglandin E_2.

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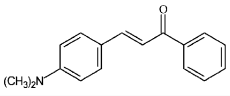
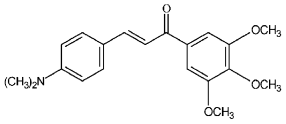
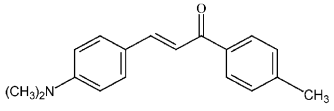
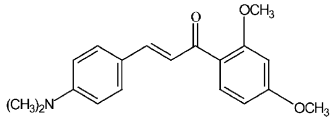
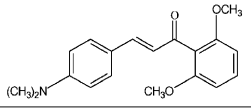
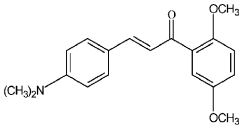
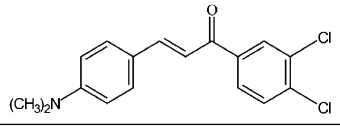
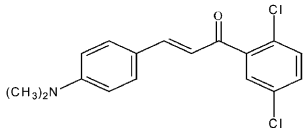
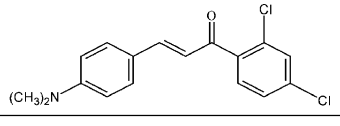
prepared by condensing aromatic aldehydes and methyl ketones to form the expected compounds, using solid sodium hydroxide as a catalyst in methanol at room temperature (Fig. 1). The starting materials were commercially available. The products obtained were formed immediately after addition of the sodium hydroxide pellet to the well stirred mixture of the aldehyde and methyl ketone as the unsaturated ketones which always yielded the *trans*-alkene (*E*-form) as judged by ^{1}H-NMR spectroscopy. Yields ranged from 80% to quantitative and were not always optimized.

3. Results and discussion

There is considerable evidence of PGE_2 and NO involvement (e.g. those generated by activated phagocytes), in processes associated with inflammation [15]. On the other hand, interest has been focused on the role of NO in septic shock [16]. Furthermore, the cytotoxic effect of NO could also cause multiple organ failure. Therefore, the inhibition of PGE_2 and NO production has been proposed as a potential therapy for different inflammatory disorders [17].

Table 1
Physicochemical properties of a series of 4-dimethylamino-chalcone derivatives

Chalcone	Structure	MW	m.p. (°C)[a]	Yield (%)[b]	Reaction time (h)
1		251	58-59	90	16
2		341	128-129	80	16
3		265	80-82	94	16
4		311	84-85	80	0.5
5		311	190-192	85	0.5
6		311	98-99	90	0.5
7		320	118-120	98	24
8		320	100-102	88	24
9		320	70-72	80	24

[a] m.p. are uncorrected.
[b] Recrystallization solvent: EtOAc/hexane (3:7).

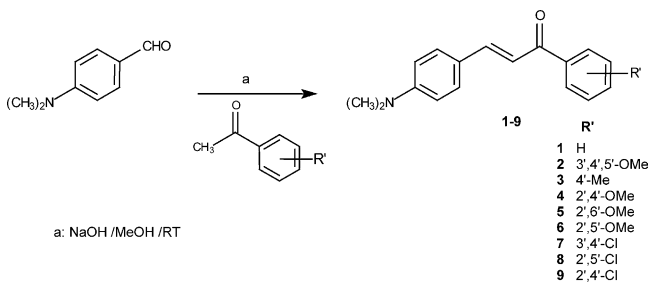
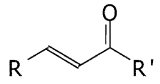
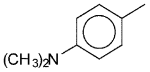
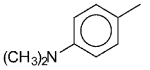
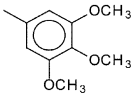
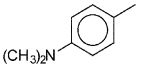
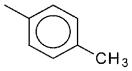
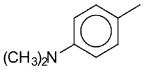
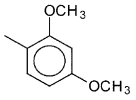
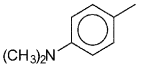
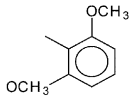
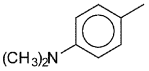
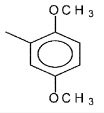
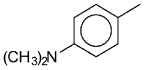
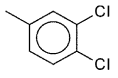
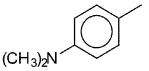
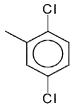
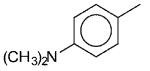
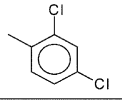


Fig. 1. General synthetic strategy employed to prepare 4-dimethylamino-chalcone derivatives.

Dimethylamino-chalcones **1**–**9** (Table 2) were studied in vitro for their inhibitory activity on the production of NO and PGE_2 mediators, produced by LPS-stimulated RAW 264.7 macrophage cells. 2′,6′ Dimethoxylation (**5**) and 2′,5′ dimethoxylation (**6**) afforded the most efficient dimethylamino-chalcone derivatives. Compounds **5** and **6** caused concentration-dependent inhibition of the production of NO with IC_{50} values of 0.7 and 0.6 μM, respectively (Fig. 2) whereas 2′,4′ dimethoxylation (**4**), lack of methoxylation (**1**), monomethylation (**3**), trimethoxylation (**2**), as well as dichlorination (**7**–**9**) led to less active or inactive compounds.

Table 2
Inhibitory activity on the production of NO and PGE_2 in RAW 264.7 macrophages of a series of 4-dimethylamino-chalcone derivatives



Chalcone	R	R′	Nitrites		PGE_2	
			% I	*IC_{50} (μM)*	*% I*	*IC_{50} (μM)*
1			49.9 ± 2.3**	-	28.7 ± 8.6*	-
2			49.1 ± 1.0**	-	36.8 ± 4.1*	-
3			32.2 ± 1.8*	-	27.9 ± 6.3*	-
4			17.1 ± 2.4	-	34.6 ± 5.3*	-
5			73.2 ± 4.1**	0.7 (0.4-1.2)	3.5 ± 3.5	-
6			63.4 ± 5.0**	0.6 (0.2-1.6)	73.3 ± 3.1**	0.9 (0.4-1.8)
7			25.4 ± 3.3	-	36.8 ± 1.7*	-
8			28.2 ± 2.6*	-	44.9 ± 1.6**	-
9			39.9 ± 2.2*	-	44.0 ± 3.3**	-
DEXAMETHASONE			***IC_{50}*** 35.8 (12.2-89.1) nM		***IC_{50}*** 1.0 (0.7-2.4) nM	

Results show mean ± SEM of percentages of inhibition at the concentration of 5 μM ($n = 6$), and IC_{50} (μM) values determined only for those compounds that reach 50% of inhibition. ** $P < 0.01$; * $P < 0.05$.

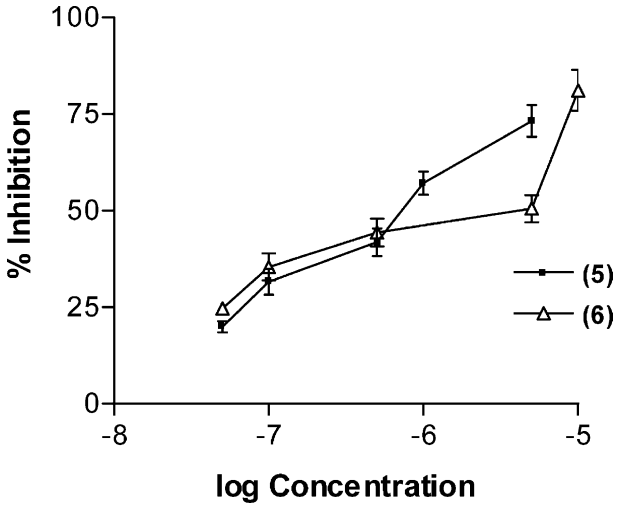


Fig. 2. Concentration-dependent inhibitory effect of chalcones **5** and **6** on nitrite production by LPS-stimulated RAW 264.7 macrophages. Results show mean ± S.E.M. of percentages of inhibition ($n = 6$). All points are statistically significant at least at $P < 0.05$.

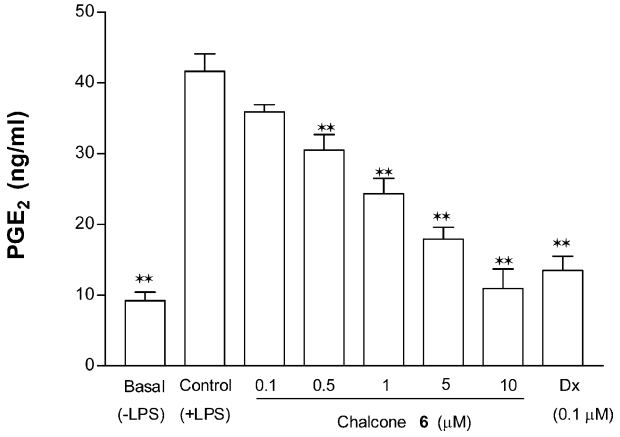


Fig. 3. Effect of chalcone **6** on PGE_2 production by LPS-stimulated RAW 264.7 macrophages. Results are the mean ± SEM of $n = 6$. ** $P < 0.01$ compared with the control group. Dx = Dexamethasone.

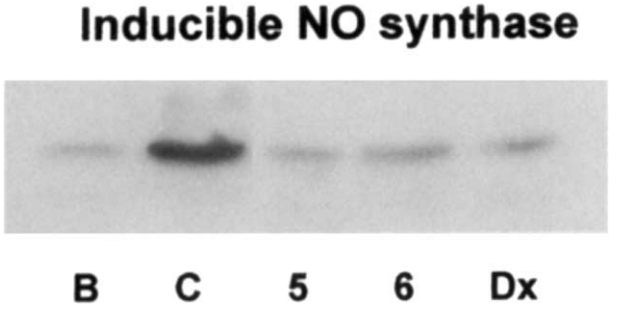


Fig. 4. Effect of chalcones **5** and **6** on inducible NO synthase expression in RAW 264.7 cells. The figure is representative of two similar experiments. B: non-stimulated cells. C: LPS-stimulated cells. 5: LPS-stimulated cells treated with chalcone **5** at 10 μM. 6: LPS-stimulated cells treated with chalcone **6** at 10 μM. Dx: LPS-stimulated cells treated with dexamethasone at 10 μM.

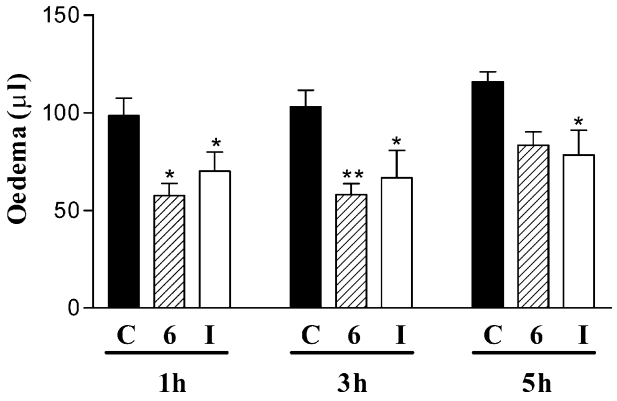


Fig. 5. Effect of chalcone **6** and indomethacin on oedema formation in the mouse paw oedema induced by carrageenan, 1, 3 and 5 h after the induction of inflammation. ** $P < 0.01$, * $P < 0.05$ with respect to the control group. C = control, 6 = chalcone **6**, I = indomethacin.

The most potent and selective inhibitor of PGE_2 production was the 2′,5′ dimethoxylated chalcone (**6**) which exhibited a dose-dependent inhibitory profile (Fig. 3) being less potent than the reference drug, dexamethasone. None of the dimethylamino-chalcone derivatives affected the cellular viability, as assessed by mitochondrial reduction of MTT after 18 h treatment on mouse macrophage cell line 264.7 (data not shown). This behaviour indicates that these chalcone derivatives were not cytotoxic.

To establish whether the inhibition of nitrite and PGE_2 production was due to an interaction with the enzyme induction process by LPS or due to a direct action of the dimethylamino-chalcone derivatives on inducible NO synthase and cyclooxygenase-2 activities, compounds **5** and **6** were added to cells which previously had expressed inducible NO synthase and cyclooxygenase-2. None of these compounds produced a significant reduction of nitrite and PGE_2 production in the post-induction phase (data not shown). This behaviour confirms that compounds **5** and **6** could display their inhibitory profile against NO and PGE_2 production by acting as inhibitors of inducible NO synthase and cyclooxygenase-2 expression. Western blot analysis was performed on lysated macrophages, obtained as previously described, to corroborate this point. As shown (Fig. 4), the increase in iNOS expression, induced by LPS, was abolished by chalcones **5** and **6** and the reference compound dexamethasone. In addition, oral administration of chalcone **6** at 25 mg kg^{-1} inhibited significantly the formation of oedema (Fig. 5) in the carrageenan-induced model of inflammation in mice. Its anti-oedematogenic activity was similar to that of indomethacin at 5 mg kg^{-1} at 3 and 5 h after carrageenan administration.

These dimethylamino-chalcone derivatives, able to control NO and PGE_2 production by affecting the

expression of those inducible enzymes involved in their production, could be an important strategy to obtain promising anti-inflammatory agents.

4. Experimental

4.1. General procedure for the synthesis of dimethylamino-chalcone derivatives

Melting points were measured on a Thomas–Hoover Unimelt apparatus and are uncorrected. Thin layer chromatography (TLC, silica gel 60 GF_{254}, Merck, Darmstadt) was used to monitor reactions and check product homogeneity. IR spectra were recorded as KBr pellets using a Shimadzu model 470 spectrometer. Nuclear Magnetic Resonance ^{1}H-NMR spectra were recorded on a JEOL GSX 270 MHz spectrometer (tetramethylsilane as internal standard). Splitting patterns are described as singlet (s), doublet (d), triplet (t), quartet (q) and multiplet (m). NMR values are given in δ units relative to $CDCl_3$. Mass spectrometry (m/z) spectra were obtained using a Hewlett Packard HP 5971A with Mass Selective Detector. Elemental analyses were performed by Atlantic Microlab Inc., Norcross, GA. Results were within $\pm 0.4\%$ of predicted values for all compounds.

A substituted methyl ketone (1 mmol), and a substituted aldehyde (1 mmol) were dissolved in a minimum amount of methanol (normally 2–4 ml). A single NaOH pellet (about 100 mg) was then added to this solution. The reaction mixture was stirred at r.t. In most cases, off-white to bright yellow solids were formed within a few minutes to 24 h (Table 1). The solids were collected on a filter and washed with cold methanol. Chalcones were recrystallized using EtOAc/hexane (3:7).

4.1.1. 4-Dimethylamino-chalcone (1)

Yield 90%, m.p.: 58–59 °C. IR (KBr): 1644 (C=O), 1568 (C=C) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 7.96 (m, 2H, H-2′, H-6′), 7.55 (m, 3H, H-3′, H-4′, H-5′) 7.43 (d, 1H, $J = 16$ Hz, H-β), 7.29 (d, 1H, $J = 16$ Hz, H-α), 6.75 (dd, 1H, $J = 8.7$, 1.8 Hz, H-6), 6.72 (dd, 1H, $J = 8.7$, 1.8 Hz, H-2), 6.57 (dd, 1H, $J = 8.7$, 2.6 Hz, H-5), 6.55 (dd, 1H, $J = 8.7$, 2.6 Hz, H-3), 2.83 (s, 6H, $N(CH_3)_2$). m/z 252 [M + 1]. Anal. $C_{17}H_{17}NO$.

4.1.2. 4-Dimethylamino-3′,4′,5′-trimethoxychalcone (2)

Yield 80%, m.p.: 128–129 °C. IR (KBr): 1644 (C=O), 1590 (C=C) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ:7.46 (d, 1H, 15.59 Hz, H-β), 7.29 (d, 1H, $J = 15.59$ Hz, H-α), 7.13 (d, 2H, H-2′, H-5′), 6.72 (dd, 1H, $J = 8.7$, 1.88 Hz, H-6), 6.71 (dd, 1H, $J = 8.7$, 1.88 Hz, H-2), 6.55 (dd, 2H, $J = 8.7$, 2.67 Hz, H-3, H-5), 3.93 (s, 9H, $(OCH_3)_2$). m/z 342 [M + 1]. Anal. $C_{20}H_{23}NO_4$.

4.1.3. 4-Dimethylamino-4′-methylchalcone (3)

Yield 94%, m.p.: 80–81 °C. IR (KBr): 1654 (C=O), 1595 (C=C) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 7.94 (dd, 2H, $J = 8.11$, 2.02 Hz, H-2′, H-6′), 7.42 (d, 1H, $J = 15.83$ Hz, H-β), 7.60 (d, 1H, $J = 15.83$ Hz, H-α), 7.34 (dd, 2H, $J = 8.11$, 2.2 Hz, H-3′, H-5′), 6.75 (dd, 1H, $J = 8.7$, 1.88 Hz, H-6), 6.72 (dd, 1H, $J = 8.7$, 1.88 Hz, H-2), 6.55 (dd, 2H, $J = 8.7$, 2.6 Hz, H-3, H-5), 2.83 (s, 6H, $N(CH_3)_2$), 2.45 (s, 3H, CH_3). m/z 266 [M + 1]. Anal. $C_{18}H_{19}NO$.

4.1.4. 4-Dimethylamino-2′,4′-dimethoxychalcone (4)

Yield 80%, m.p.: 84–85 °C. IR (KBr): 1644 (C=O), 1590 (C=C) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ:7.68 (d, 2H, $J = 8.66$, H-3, H-5), 7.64 (d, 1H, $J = 15.59$ Hz, H-β), 7.47 (d, 2H, $J = 8.91$ Hz, H-2, H-6), 7.26 (d, 1H, $J = 15.83$ Hz, H-α), 6.64 (d, 1H, 8.91 Hz, H-6′), 6.51 (dd, 1H, $J = 2.99$, 2.23 Hz, H-3′), 6.48 (dd, 1H, $J =$ 8.91, 2.2 Hz, H-5′), 3.86 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3), 3.00 (s, 6H, $N(CH_3)_2$). m/z 312 [M + 1]. Anal. $C_{19}H_{21}NO_3$.

4.1.5. 4-Dimethylamino-2′,6′-dimethoxychalcone (5)

Yield 85%, m.p.: 190–101 °C. IR (KBr): 1644 (C=O), 1590 (C=C) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 7.39 (d, 2H, $J = 8.9$ Hz, H-3, H-5), 7.30 (t, 1H, $J = 8.41$ Hz, H-4′), 7.26 (*d*, 1H, $J = 16.82$ Hz, H-β), 6.76 (d, 1H, $J = 16.82$ Hz, H-α), 6.62 (d, 2H, $J = 10.64$ Hz, H-5′, H-3′), 6.58 (d, 2H, $J = 8.41$ Hz, H-2, H-6), 3.7 (s, 6H, $(OCH_3)_2$, 2.98 (s, 6H, $N(CH_3)_2$). m/z 312 [M + 1]. Anal. $C_{19}H_{21}NO_3$.

4.1.6. 4-Dimethylamino-2′,5′-dimethoxychalcone (6)

Yield 90%, m.p.: 190–101 °C. IR (KBr): 1644 (C=O), 1590 (C=C) cm − 1. ^{1}H-NMR ($CDCl_3$) δ: 7.55 (d, 1H, $J = 15.83$ Hz, H-β), 7.46 (d, 2H, $J = 8.66$ Hz, H-3′, H-4′), 7.30 (d, 1H, $J = 15.83$ Hz, H-α), 7.11 (d, H, $J = 2.2$ Hz, H-6′), 6.96 (dd, 2H, $J = 8.91$, 3.22 Hz, H-2, H-6),), 6.92 (d, 1H, $J = 8.91$ Hz, H-3), 6.65 (d, 1H, $J = 8.91$ Hz, H-5), 3.81 (s, 3H, OCH_3), 3.78 (s, 3H, OCH_3), 2.98 (s, 6H, $N(CH_3)_2$, m/z 312 [M + 1]. Anal. $C_{19}H_{21}NO_3$.

4.1.7. 4-Dimethylamino-3′,4′-dichlorochalcone (7)

Yield 98%, m.p.: 118–119 °C. IR (KBr): 1644 (C=O), 1590 (C=C) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 8.06 (d, 1H, $J = 1.98$ Hz, H-2′), 7.82-7.76 (m, 2H, H-β, H-6′), 7.52 (d, 4H, $J = 9.40$, H-2, H-3, H-6, H-5), 7,21 (d, 1H, $J = 15.59$, H-α), 6.67 (d, 1H, $J = 8.91$ Hz, H-5′), 2.98 (s, 6H, $N(CH_3)_2$). Anal. $C_{17}H_{15}Cl_2NO$.

4.1.8. 4-Dimethylamino-2′,5′-dichlorochalcone (8)

Yield 88%, m.p.: 100–101 °C. IR (KBr): 1644 (C=O), 1590 (C=C) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 7.93 (s, 1H, H.6′), 7.42–7.35 (m, 4H, H-2, H-3, H-5, H-β), 7.32 (d, 2H, $J = 8.1$ Hz, H-3′, H-4′), 7.31–6.62 (m, 3H, H-α,

H-6), 2.98 (s, 6H, $N(CH_3)_2$). *m/z* 321 [M + 1]. Anal. $C_{17}H_{15}Cl_2NO$.

*4.1.9. 4-Dimethylamino-2′,4′-dichlorochalcone (**9**)*

Yield 80%, m.p.: 70–72 °C. IR (KBr): 1644 (C=O), 1580 (C=C) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 7.49–7.32 (m, 5H, 4H Ar, H-β). 7.31 (d, 1H, $J = 2.08$ Hz, H-3′), 6.85 (d, 1H, $J = 16.2$ Hz, H-α), 6.66 (d, 2H, $J = 8.1$ Hz, H-6′, H-6), *m/z* 321 [M + 1]. Anal. $C_{17}H_{15}Cl_2NO$.

4.2. Pharmacology

4.2.1. Materials

For the in vitro assays [5,6,8,11,12,14,15(n)-^{3}H]prostaglandin E_2 was purchased from Amersham Iberica, (Madrid, Spain). The rest of reagents were from Sigma Chemical Co. (St. Louis, MO).

4.2.2. Cell culture

The mouse macrophage cell line RAW 264.7 (European Collection of Cell Cultures) was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 2 mM L-glutamine, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 10% fetal bovine serum. Macrophages were removed from the tissue culture flask using a cell scraper and centrifuged at 400 × *g* for 10 min. Cells were resuspended at a concentration of 2×10^6 cells ml^{-1} in a total volume of 200 μl and cultured in 96-well culture plates.

4.2.3. Cell viability assays

The mitochondrial dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan [18] was used to assess the possible cytotoxic effect of dimethylamino-chalcone derivatives on the mouse macrophage cell line RAW 264.7.

4.2.4. Inducible NO synthase and cyclooxygenase-2 activity in intact cells

Macrophages were co-incubated with test compounds and *Escherichia coli* LPS (serotype 0111:B4) (10 μg ml^{-1}) at 37 °C for 18 h. Nitrite concentration as reflection of NO release was assayed fluorometrically [19]. The amount of nitrite was obtained by extrapolation from a standard curve with sodium nitrite as a standard. PGE_2 levels were measured by radioimmunoassay [20] as an index of cyclooxygenase-2 activity.

4.2.5. Western blot analysis

Inducible NO synthase protein expression was studied in the cytosolic or microsomal fractions, respectively, from LPS-stimulated RAW 264.7 cells. Equal amounts of protein (20 μg) were loaded on 12.5% polyacrylamide gel electrophoresis–sodium dodecyl sulphate (PAGE–SDS) and transferred onto polyvinylidene difluoride membranes for 90 min at 125 mA. Membranes were blocked in phosphate buffer saline (0.02 M, pH 7.0)–Tween 20 (0.1%) containing 3% w/v unfatted milk. For inducible NO synthase, membranes were incubated with specific anti-inducible NO synthase polyclonal antiserum (1/1000); for cyclo-oxygenase-2, membranes were incubated with specific anti-cyclo-oxygenase-2 polyclonal antiserum (1/1000). Both membranes were incubated with the peroxidase-conjugated goat anti-rabbit immunoglobulin G (1/20,000) and peroxidase-conjugated rabbit anti-goat/sheep IgG (1/20,000), respectively. The immunoreactive bands were visualized using an enhanced chemiluminescence system.

4.2.6. Mouse paw oedema

The anti-inflammatory activity of chalcone **6** was assessed by the carrageenan paw oedema test in mice according to the method of Sugishita et al. [21]. Chalcone 6 (25 mg kg^{-1}), indomethacin (5 mg kg^{-1}) or vehicle (Tween-80/ethanol/water 5:5:90, v/v) was administered orally 1 h before the injection of carrageenan (0.05 ml; 3% w/v in saline) into the subplantar area of the right hind paws of groups of six animals. The volumes of injected and contralateral paws were measured at 1, 3 and 5 h after induction of oedema by using a plethysmometer (Ugo Basile, Comerio, Italy). The volume of oedema was expressed for each animal as the difference between the carrageenan-injected and contralateral paws.

4.2.7. Statistical analysis

The results are presented as means ± S.E.M.; *n* represents the number of experiments. Inhibitory concentration 50% (IC_{50}) values were calculated from at least four significant concentrations ($n = 6$). The level of statistical significance was determined by analysis of variance (ANOVA) followed by Dunnett's *t*-test for multiple comparisons.

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