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Synthesis and Anti-inflammatory Effect of Chalcones

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Abstract

The process of degranulation of mast cells and neutrophils contributes to inflammatory disorders. Activation of microglial cells and macrophages is believed to be involved in inflammatory, infectious and degenerative diseases of the CNS. Combining the potent inhibition of chemical mediators released by the degranulation of mast cells or neutrophils and from the activated microglial cells or macrophages, would lead to a promising anti-inflammatory agent for the treatment of peripheral and central inflammation. A series of chalcone derivatives have been reported to have potent anti-inflammatory activity. In an effort to continually develop potent anti-inflammatory agents, novel series of chalcones, 2′-hydroxy- and 2′,5′-dihydroxychalcones were synthesized and their inhibitory effects on the activation of mast cells, neutrophils, microglial cells and macrophages were evaluated in-vitro. The chalcones were prepared by Claisen-Schmidt condensation of appropriate acetophenones with an appropriate aromatic aldehyde. The alkoxychalcones were prepared with appropriate hydroxychalcones and alkyl iodide and the dihydroxychalcones were prepared by hydrogenation of an appropriate chalcone with Pd/C.

Almost all of the hydroxychalcones exhibited potent inhibitory effects on the release of β-glucuronidase and lysozyme from rat neutrophils stimulated with formyl-Met-Leu-Phe/cytochalasin B (fMLP/CB). Of the hydroxychalcones, compound **1** was the most potent inhibitor of the release of β-glucuronidase (IC50=$1{\cdot}6 \pm 0{\cdot}2\ \mu$M) and lysozyme (IC50=$1{\cdot}4 \pm 0{\cdot}2\ \mu$M) from rat neutrophils stimulated with fMLP/CB. Almost all of the 2′,5′-dialkoxychalcones exhibited potent inhibitory effects on nitric oxide (NO) formation from murine microglial cell lines N9 stimulated with lipopolysaccharide (LPS). Of these, compound **11** showed the greatest effect (IC50=$0{\cdot}7 \pm 0{\cdot}06\ \mu$M).

The present results demonstrated that most of the chalcone derivatives have an anti-inflammatory effect. The inhibitory effects of dialkoxychalcones, **10**–**12** on inflammation are probably not due to the inhibition of mast cells and neutrophil degranulation, but are mediated through the suppression of NO formation from N9 cells.

The processes of degranulation of mast cells and neutrophils contributes to inflammatory disorders (Middleton & Kandaswami 1992). Combining potent inhibition of chemical mediators released from mast cell and neutrophil degranulation, would suggest a promising anti-inflammatory agent. Our previous reports indicated that some chalcones exert potent anti-inflammatory effects, at least partly, through the suppression of chemical mediators released from mast cell and neutrophil degranulation (Lin et al 1997; Hsieh et al 1998). These findings suggested that some chalcones might be promising anti-inflammatory agents. In our current study, we further synthesize and describe the anti-inflammatory effects of chalcones using in-vitro inflammatory models.

Macrophages are important in nonspecific host resistance to microbial pathogens and serve as central regulators of the specific immune response (Solbach et al 1991). Upon activation, nitric oxide (NO), together with other chemical mediators, is released in response to bacterial endotoxin (lipopolysaccharide, LPS) (Ding et al 1988). NO plays a central role in macrophage-induced cytotoxicity,

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however; excess NO may contribute to the pathophysiology of septic shock (Thiermermann & Vane 1990). Microglial cells, the brain's resident macrophages, have detrimental as well as beneficial effects on the surrounding cells and are believed to be involved in most inflammatory, infectious, and degenerative diseases of the CNS (Mallat & Chamak 1994; Gehrmann et al 1995). The release of NO by activated microglial cells in response to LPS is likely to have a crucial role in mediating the interaction between microglia and other cells present in the nervous system, as it is known to regulate inflammation, immune functions, blood vessel dilation, neurotransmission and neural-cell survival (Gebicke-Haerter et al 1989; Nathan 1992; Minghetti et al 1997). Hence, inhibition of NO released from microglial cells or macrophages is a rational therapeutic approach to treat a variety of inflammatory and neuronal conditions observed in ageing and Alzheimer's disease (Meda et al 1995).

In this study, the anti-inflammatory activities of a series of synthetic compounds were assessed in-vitro by determining their inhibitory effects on macrophages and microglial cells, as well as on mast cells and neutrophils, and their structure–activity relationships are discussed.

Materials and Methods

Mast cell degranulation

Heparinized Tyrode's solution was injected into the peritoneal cavity of exsanguinated rats (Sprague-Dawley, 250–300 g). After abdominal massage, cells in the peritoneal fluid were harvested and then separated in 38% bovine serum albumin (BSA) in glucose-free Tyrode's solution. Cell pellets were washed and suspended in Tyrode's solution with 0·1% BSA to 1×10^6 cell mL^{-1}. The cell suspension was then pre-incubated at 37°C with 0·5% dimethylsulphoxide (DMSO) or drugs for 3 min. Fifteen minutes after the addition of compound 48/80 (10 $\mu g\,mL^{-1}$), β-glucuronidase (phenolphthalein-β-glucuronide as substrate, 550 nm) and histamines (*o*-phthadialdehyde condensation, 350/450 nm) in the supernatant were determined (Wang et al 1994). The total content was measured after treatment of the cell suspension with Triton X-100 and the percentage released was calculated. The final concentration of drugs in DMSO was fixed at 0·5%.

Neutrophil degranulation

Blood was withdrawn from rat and mixed with EDTA. After dextran sedimentation, Ficoll-Hypaque separation and hypotonic lysis of the residual erythrocytes, neutrophils were washed and suspended in Hank's balanced salt solution (HBSS) to 1×10^7 cells mL^{-1} (Boyum 1968). The cell suspension was pre-incubated at 37°C with 0·5% DMSO or drugs for 3 min, and then challenged with formyl-Met-Leu-Phe/cytochalasin B (fMLP/CB; 1 μM/5 $\mu g\,mL^{-1}$). After 45 min, the lysozyme (*Micrococcus lysodeikticus* as substrate, 450 nm) and β-glucuronidase in the supernatant were determined (Smith & Iden 1979). The total content was measured after treatment of the cell suspension with Triton X-100 and the percentage released was calculated. The final concentration of drugs in DMSO was fixed at 0·5%.

Superoxide anion formation

Superoxide anion formation was measured in terms of superoxide dismutase-inhibitable cytochrome *c* reduction (Market et al 1984). Neutrophil suspension was pre-incubated with 0·5% DMSO or drugs for 3 min, and then superoxide dismutase or HBSS was added into the test and blank wells, respectively. After addition of cytochrome *c*, the reaction was initiated by challenge with fMLP/CB (0·3 μM/5 $\mu g\,mL^{-1}$) or phorbol 12-myristate B acetate (PMA) (3 nM). The reaction was terminated after 30 min by centrifugation and the absorbance changes of supernatant were monitored at 550 nm in a microplate reader. The final concentration of drugs in DMSO was fixed at 0·5%.

Macrophage cultures and drugs treatment

Raw 264·7 mouse macrophage-like cell line (American Type Culture Collection) was plated in 96-well tissue-culture plates in Dulbecco's Modified Eagle Medium supplemented with 5% foetal calf serum (FCS), 100 int. units mL^{-1} of penicillin and streptomycin at 2×10^5 cells per 200 μL per well. Cells were allowed to adhere overnight. Pretreatment of cells with test drugs at 37°C for 1 h before stimulation with 1 $\mu g\,mL^{-1}$ of LPS (*Escherichia coli*, serotype 0111 : B4) for 24 h, and then the medium was collected and stored at −70°C until used. The final concentration of drugs in DMSO was fixed at 0·5%.

Microglial cell cultures and drugs treatment

Murine microglial cell lines N9 (Corradin et al 1993) (kindly provided by Dr P. Ricciardi-Castagnoli, CNR Cellular and Molecular Pharmacology Center, Italy) was plated in 96-well tissue-culture plates in Iscove's Modified Dulbecco's medium containing 2% heat-inactivated FCS and antibiotics at 8×10^4 cells per 200 μL^{-1} per

well. Pretreatment of cells with test drugs at 37°C for 1 h before stimulation with LPS (10 μg mL^{-1}) for 24 h, and then the medium was collected and stored at −70°C until used. The final concentration of drugs in DMSO was fixed at 0·5%.

NO determination

The production of NO was determined in cell medium by measuring the content of nitrite (Minghetti et al 1997) based on the Griess reaction. Briefly, 40 μL of 5 mM sulphanilamide, 10 μL of 2 M HCl and 20 μL of 40 mM naphthylethylenediamine were added to 150 μL culture medium. After 10 min of incubation at room temperature, absorbance was measured at 550 nm in a microplate reader. A standard nitrite curve was generated in the same fashion using $NaNO_2$. The final concentration of drugs in DMSO was fixed at 0·5%.

Statistical analysis

Data are presented as the means ± s.e.m. Statistical analyses were performed using the Least Significant Difference Test method after analysis of variance. $P < 0{\cdot}05$ was considered to be significant. Analysis of the regression line was used to calculate IC50 values.

Chemistry

Melting points (uncorrected) were determined with a Yanaco Micro-Melting Point apparatus. IR spectra were determined with a Hitachi model 260-30 IR spectrophotometer. ^{1}H and ^{13}C NMR spectra (δ, ppm; J, Hz) (Tables 2 and 3) were determined with a Varian Gemini 200 MHz FT-NMR spectrometer. Mass spectra were determined with a Jeol JMS-D-100 mass spectrometer. Elemental analyses were within ±0·4% of the theoretical values, unless otherwise noted. Chromatography was performed using a flash-column technique on silica gel 60 supplied by E. Merck.

We prepared a number of new chalcones (**1**–**9**; Table 1) using Claisen-Schmidt condensation of appropriate acetophenones or hydroxyacetophenones, protected as tetrahydropyranyl ether, with an appropriate aromatic aldehyde or hydroxyaromatic aldehyde, protected as tetrahydropyranyl ether (Hsieh et al 1998). A mixture of 2′,5′-dihydroxy-4-chlorochalcone (**16**), appropriate alkyl iodide and K_2CO_3 in *N,N*-dimethylformamide

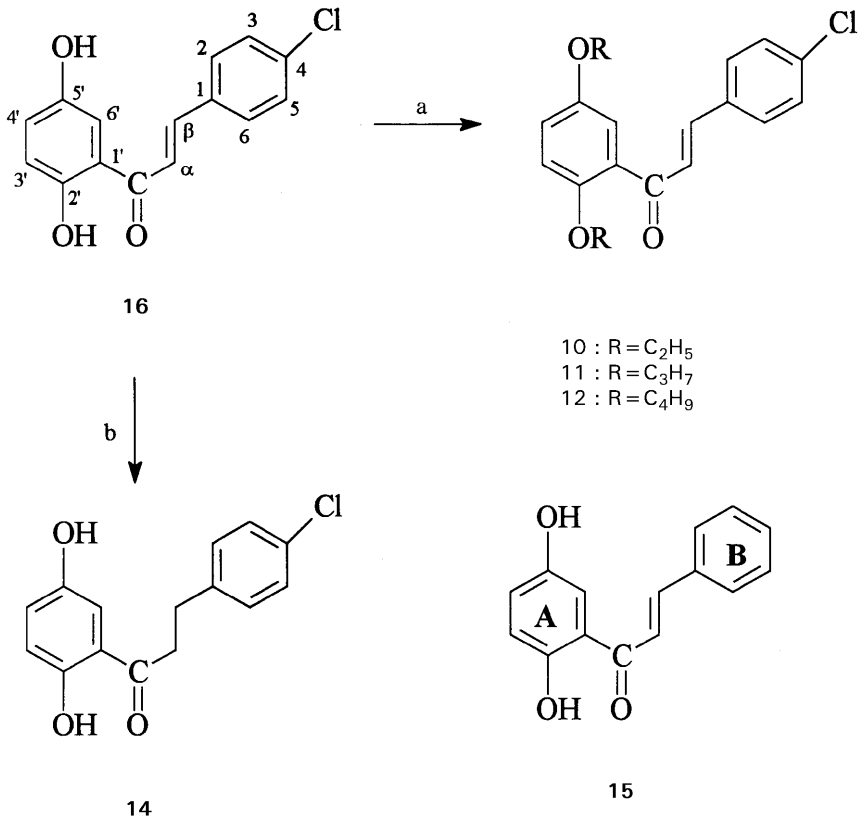
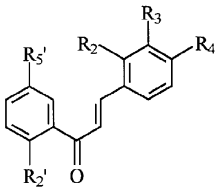
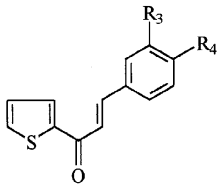


Figure 1. Synthesis of 2′,5′-dihydroxy-4-chlorodihdrochalcone (**14**) and general synthesis of 2′,5′-dialkoxychalcones **10**–**12**. (a) Alkyl halide, K_2CO_3, DMF, room temperature; (b) EtOAc, H_2, 5% Pd/C, room temperature.

Table 1. Chalcones **1**–**12** and dihydrochalcones **13**–**14**.



1,2,4-12



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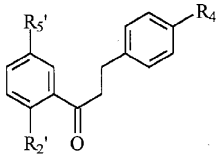

13,14

Compound	R2′	R5′	R2	R3	R4	mp (°C)	% Yield	Formula	Anal.
1	OH	H	OH	H	H	160–162	46[a]	$C_{15}H_{12}O_3$	C, H
2	OH	H	H	H	Cl	160–161	38[b]	$C_{15}H_{11}O_2Cl$	C, H
3				OH	OH	183–185	38[c]	$C_{13}H_{10}O_3S$	C, H
4	OCH_3	OCH_3	H	OH	OH	158–160	48[d]	$C_{17}H_{16}O_5$	C, H
5	OH	OCH_3	H	OCH_3	OCH_3	131–132	42[b]	$C_{18}H_{18}O_5$	C, H
6	OH	OCH_3	H	H	OCH_3	87–88	46[b]	$C_{17}H_{16}O_4$	C, H
7	OH	OCH_3	H	H	CH_3	93–94	52[b]	$C_{17}H_{16}O_3$	C, H
8	OH	OCH_3	H	H	Cl	116–117	34[b]	$C_{16}H_{13}O_3Cl$	C, H
9	OCH_3	OCH_3	H	H	Cl	90–91	42[b]	$C_{17}H_{15}O_3Cl$	C, H
10	OC_2H_5	OC_2H_5	H	H	Cl	115–116	62[b]	$C_{19}H_{19}O_3Cl$	C, H
11	OC_3H_7	OC_3H_7	H	H	Cl	70–71	60[b]	$C_{21}H_{23}O_3Cl$	C, H
12	OC_4H_9	OC_4H_9	H	H	Cl	72–73	52[b]	$C_{23}H_{27}O_3Cl$	C, H
13	OH	OH	H	H	H	115–116	82[e]	$C_{15}H_{14}O_3$	C, H
14	OH	OH	H	H	Cl	131–132	88[e]	$C_{15}H_{13}O_3Cl$	C, H

Solvents used for recrystallization: [a]Cyclohexane–ethyl acetate. [b]Methanol. [c]Benzene. [d]Cyclohexane–acetone. [e]Benzene–petroleum ether.

(DMF) was stirred at room temperature to give compounds **10**–**12** (Figure 1; Table 1). A solution of dihydroxychalcone or **16** in EtOAc was hydrogenated with Pd/C at room temperature to give **13** and **14** (Figure 1), respectively (Table 1).

*General procedure for obtaining chalcones **1**–**9** (Hsieh et al 1998)*

*2′-Thienyl-3,4-dihydroxychalcone (**3**).* 3,4-Dihydroxybenzaldehyde (3·45 g, 25 mmol) and pyridinium *p*-toluenesulphonate (0·15 g, 0·6 mmol) were stirred for 0·5 h in methylene chloride (100 mL) and then 3,4-dihydro-α-pyran in methylene chloride (13 mL in 20 mL) was added dropwise. The reaction mixture was stirred at room temperature for 4 h, washed twice with water, dried and evaporated in-vacuo. The residue yielded crude 3,4-bis(tetrahydropyran-2-yloxy)benzaldehyde (**3a**). Crude **3a**, 2-thiophenecarboxacetophenone (3·15 g, 25 mmol) and barium hydroxide octahydrate (8·15 g, 25 mmol) were dissolved in MeOH (100 mL). The reaction mixture was stirred for 12 h at 40°C and then evaporated in-vacuo. Water (100 mL) was added and the mixture was neutralized with HCl (1 M, 35 mL) and extracted with EtOAc. The organic layer was separated, washed with water, dried and evaporated in-vacuo. The residue yielded crude 2′-thienyl-3,4-bis(tetrahydropyran-2-yloxy)-chalcone (**3b**). Crude **3b** and *p*-toluenesulphonic acid (0·2 g, 1·05 mmol) were dissolved in MeOH (100 mL). The reaction mixture was stirred for 4 h at room temperature, and then evaporated in-vacuo. Water (100 mL) was added to the mixture, neutralized with 5% $NaHCO_3$ (50 mL) and extracted with EtOAc. The organic layer was separated, washed with water, dried and evaporated in-vacuo. The residue was eluted through a silica-gel column with cyclohexane–EtOAc (4 : 1) to give **3** (2·3 g, 9·5 mmol, 38%): IR (KBr) 3200, 1635 cm^{-1}; MS m/z 246 (M^+, 111).

*2′,2-Dihydroxychalcone (**1**).* 2-Hydroxyacetophenone (3·4 g, 25 mmol), 2-hydroxybenzaldehyde (3·05 g, 25 mmol) and barium hydroxide octahydrate (8·15 g, 25 mmol) were treated as in the synthesis of **3b** to give **1** (2·8 g, 11·5 mmol, 46%): IR (KBr) 3256, 1636 cm^{-1}; MS m/z 240 (M^+, 221).

*2′-Hydroxy-4-chlorochalcone (**2**).* 2-Acetophenone (3·4 g, 25 mmol), 4-chlorobenzaldehyde (3·5 g, 25 mmol) and barium hydroxide octahydrate (8·15 g, 25 mmol) were treated as in the synthesis of **3b** to give **2** (2·7 g, 9·5 mmol, 38%): IR (KBr) 1620 cm^{-1}; MS m/z 258 (M^+, 147).

*2′,5′-Dimethoxy-3,4-dihydroxychalcone (**4**).* 3,4-Dihydroxybenzaldehyde (3·45 g, 25 mmol) and pyridinium *p*-toluenesulphonate (0·15 g, 0·6 mmol)

were treated as in the synthesis of **3a** to give crude 3,4-bis(tetrahydro-pyran-2-yloxy)benzaldehyde (**4a**). Crude **4a**, 2,5-dimethoxyacetophenone (4·5 g, 25 mmol) and barium hydroxide octahydrate (8·15 g, 25 mmol) were treated as in the synthesis of **3b** to give **4** (3·6 g, 12 mmol, 48%): IR (KBr) 3400, 1610 cm^{-1}; MS m/z 300 (M^+, 77).

*2′-Hydroxy-5′,3,4-trimethoxychalcone (**5**).* 2-Hydroxy-5-methoxyacetophenone (4·15 g, 25 mmol), 3,4-dimethoxybenzaldehyde (4·2 g, 25 mmol) and barium hydroxide octahydrate (8·15 g, 25 mmol) were treated as in the synthesis of **3b** to give **5** (3·3 g, 10·5 mmol, 42%): IR (KBr) 2850, 1640 cm^{-1}; MS m/z 314 (M^+, 164).

*2′-Hydroxy-5′,4-dimethoxychalcone (**6**).* 2′-Hydroxy-5′-methoxyacetophenone (4·15 g, 25 mmol), 4-methoxybenzaldehyde (3·4 g, 25 mmol) and barium hydroxide octahydrate (8·15 g, 25 mmol) were treated as in the synthesis of **3b** to give **6** (3·2 g, 11·5 mmol, 46%): IR (KBr) 3000, 1640 cm^{-1}; MS m/z 284 (M^+, 134).

*2′-Hydroxy-5′-methoxy-4-methylchalcone (**7**).* 2-Hydroxy-5-methoxyacetophenone (4·15 g, 25 mmol), 4-methylbenzaldehyde (3 g, 25 mmol) and barium hydroxide octahydrate (8·15 g, 25 mmol) were treated as in the synthesis of **3b** to give **7** (3·5 g, 13 mmol, 52%): IR (KBr) 3100, 1640 cm^{-1}; MS m/z 268 (M^+, 150).

*2′-Hydroxy-5′-methoxy-4-chlorochalcone (**8**).* 2′-Hydroxy-5′-methoxyacetophenone (4·15 g, 25 mmol), 4-chlorobenzaldehyde (3·5 g, 25 mmol) and barium hydroxide octahydrate (8·15 g, 25 mmol) were treated as in the synthesis of **3b** to give **8** (2·4 g, 8·5 mmol, 34%): IR (KBr) 3100, 1610 cm^{-1}; MS m/z 288 (M^+, 150).

*2′,5′-Dimethoxy-4-chlorochalcone (**9**).* 2,5-Dimethoxyacetophenone (3·75 g, 25 mmol), 4-chlorobenzaldehyde (3·5 g, 25 mmol) and barium hydroxide octahydrate (8·15 g, 25 mmol) were treated as in the synthesis of **3b** to give **9** (3·1 g, 10·5 mmol, 42%): IR (KBr) 3100, 1645 cm^{-1}; MS m/z 302 (M^+, 165).

*General procedure for obtaining alkoxychalcones **10–12***

*2′,5′-Diethoxy-4-chlorochalcone (**10**).* A mixture of 2′,5′-dihydroxychalcone (6 g, 25 mmol), ethyl iodide (8·1 g, 52 mmol) and potassium carbonate (15 g, 25 mmol) in DMF (50 mL) was stirred at room temperature for 18 h. The mixture was diluted with water and washed three times with water. The organic phase was dried over sodium sulphate, filtered and concentrated in-vacuo to give the product. Purification via silica-gel column chromatography provided 5·15 g (15·5 mmol, 62%) of **10** as a yellow microcrystalline solid: IR (KBr) 2980, 1640 cm^{-1}; MS m/z 330 (M^+, 164).

*2′,5′-Dipropoxy-4-chlorochalcone (**11**).* 2′,5′-Dihydroxychalcone (6 g, 25 mmol), n-propyl iodide (8·8 g, 52 mmol) and potassium carbonate (15 g, 25 mmol) in DMF (50 mL) were treated as in the synthesis of **10** to give **11** (5·37 g, 15 mmol, 60%): IR (KBr) 3050, 1620 cm^{-1}; MS m/z 358 (M^+, 136).

*2′,5′-Dibutoxy-4-chlorochalcone (**12**).* 2′,5′-Dihydroxychalcone (6 g, 25 mmol), n-butyl iodide (9·5 g, 52 mmol) and potassium carbonate (8·15 g, 25 mmol) in DMF (50 mL) were treated as in the synthesis of **10** to give **12** (5 g, 13 mmol, 52%): IR (KBr) 2980, 1620 cm^{-1}; MS m/z 386 (M^+, 136).

*General procedure for obtaining dihydrochalcones **13–14***

*2′,5′-Dihydroxydihydrochalcone (**13**).* A solution of 2′,5′-dihydroxychalcone (1·7 g, 7 mmol) in ethyl acetate (50 mL) was hydrogenated in an autoclave for 3 h at an initial pressure of 60 kg cm^{-3} in the presence of 5% Pd/C (300 mg) at room temperature. The catalyst was removed by filtration and the filtrate was evaporated in-vacuo and purified by chromatography over a silica-gel column using a 5 : 1 mixture of cyclohexane and ethyl acetate as eluant to afford the crude product. The crude product was recrystallized from benzene–petroleum ether to give **13** (1·4 g, 5·74 mmol, 82%): IR (KBr) 3400, 1630 cm^{-1}; MS m/z 242 (M^+, 137).

*2′,5′-Dihydroxy-4-chlorodihydrochalcone (**14**).* 2′,5′-Dihydroxy-4-chlorochalcone (2 g, 7 mmol) and 5% Pd/C (300 mg) in ethyl acetate were treated as in the synthesis of **13** to give **14** (1·7 g, 6·2 mmol, 88%): IR (KBr) 3440, 1660 cm^{-1}; MS m/z 276 (M^+, 137).

Results and Discussion

The anti-inflammatory activities of **1–14** (Table 1) were studied in-vitro for their inhibitory effects on chemical mediators released from mast cells, neutrophils, macrophages and microglial cells. Compounds **1**, **5–7** and **9** caused concentration-

Table 2. ^{1}H NMR data for various chalcone and dihydrochalcone derivatives.

Compound	H-6′	H-3′	H-5′	H-4′	H-2	H-6	H-3	H-5
1[a]	8·20(dd)		6·90–7·04(m)	7·27–7·36(m)		7·87(dd)		6·90–7·04(m)
2[b]	7·91(dd)			7·39–7·62(m)				6·91–7·06(m)
3[c]	7·10(d)	8·02(dd)	7·84(dd)	7·22(dd)	7·19(d)	7·10(dd)		6·82(d)
4[b]	7·10(d)	6·99(d)		6·96(dd)	7·06(d)	6·98(dd)		6·79(d)
5[b]	7·39(d)	6·97(d)		7·14(dd)	7·16(d)	7·27(dd)		6·91(d)
6[b]	7·36(d)	6·97(d)		7·13(dd)				6·96(dd)
7[b]	7·36(d)	6·97(d)		7·14(dd)				7·24(m)
8[b]	7·34(d)	6·98(d)		7·16(dd)				7·40–7·45(m)
9[b]	7·19(d)	6·93(d)		7·03(dd)				7·26–7·38(m)
10[b]	7·22(d)	6·91(d)		7·02(dd)			7·37–7·56(m)	
11[b]	7·22(d)	6·90(d)		7·01(dd)		7·50–7·52(m)		7·35–7·37(m)
12[b]	7·22(d)	6·90(d)		7·02(dd)		7·51–7·74(m)		7·35–7·37(m)
13[c]	7·17(d)	6·87(d)		7·01(dd)				7·20–7·30(m)
14[c]	7·14(d)	6·87(d)		6·97(dd)			7·16–7·29(m)	

Compound	H-4	H-α	H-β	OCH_3	CH_3	CH_2	OCH_2
1[a]	7·51–7·60(m)	8·07(d)	8·34(d)				
2[b]			7·62(d)	7·87(d)			
3[c]		7·41(d)	7·68(d)				
4[b]		7·16(d)	7·44(d)	3·73;3·79			
5[b]		7·44(d)	7·88(d)	3·84;3·94;3·97			
6[b]		7·47(d)	7·90(d)	3·85;3·87			7·62(dd)
7[b]		7·54(m)	7·90(d)	3·84(s)	2·41(s)		7·54(m)
8[b]		7·55(d)	7·86(d)	3·85(s)			7·57–7·62(m)
9[b]		7·39(d)	7·58(d)	3·81;3·86			7·49–7·54(m)
10[b]		7·30(d)	7·60(d)		1·36–1·43(m)		3·98–4·13(m)
11[b]		7·50(d)	7·59(d)		1·0(m)	1·8(m)	3·91(m)
12[b]		7·50(d)	7·59(d)		0·92(m)	1·45;1·70(m)	3·97(m)
13[c]		3·04(t)	3·25(t)				
14[c]		3·04(t)	3·23(t)				

[a]Measured in $(CD_3)_2CO$. [b]Measured in $CDCl_3$. [c]Measured in CD_3OD.

dependent inhibition of the mast cell degranulation stimulated with compound 48/80 ($10\,\mu g\,mL^{-1}$) ($41{\cdot}5 \pm 1{\cdot}2$ and $63{\cdot}7 \pm 2{\cdot}7\%$ release of β-glucuronidase and histamine, respectively, as control values) (Table 4), indicating that substituting the 2′,5′-diphenolic or 2′,5′-dihydroxyphenyl or the B ring of 2′,5′-dihydroxychalcone did not enhance the inhibitory effects on mast cells degranulation (Hsieh et al 1998). The finding that reduction of **15** to the 2′,5′-dihydroxydihydrochalcone (**13**) abolished the inhibitory activity (Table 4) indicates that the enone moiety of chalcones appears to be required for the inhibition of mast cell degranulation. Mepacrine was used in this study as a positive control and produced a dose-dependent inhibition of mast cell degranulation caused by compound 48/80.

fMLP/CB ($1\,\mu M/5\,\mu g\,mL^{-1}$) induced the release of β-glucuronidase and lysozyme ($21{\cdot}1 \pm 1{\cdot}5$ and $40{\cdot}6 \pm 2{\cdot}0\%$, respectively) from rat neutrophils. Compounds **1**, **15** and **16** had potent and concentration-dependent inhibitory effects on the activation of neutrophils while **2** did not have any significant inhibitory effects (Table 5). Compounds **4**–**7** also had significant and concentration-dependent inhibitory effects on the activation of neutrophils (Table 5). Compound **9** showed significant inhibitory effects on the activation of neutrophils while **8** did not. This clearly indicates that a 2′-hydroxy- or 2′,5′-dihydroxychalcone with an appropriate substitution in its B ring shows potent inhibitory effects on the activation of neutrophils. *O*-methylation of **16** slightly decreased the inhibition of fMLP/CB-induced neutrophil degranulation, whereas with other types of *O*-alkylation of **16** it was abolished (Table 5), indicating that the increase in lipophicity of **16** did not enhance the inhibitory activity. Reduction of **15** and **16** to **13** and **14**, respectively, was also shown not to enhance the inhibitory effects on the fMLP/CB-induced response (Hsieh et al 1998). Hence the enone moiety of chalcones appears to be required for inhibition of neutrophil degranulation. Trifluoperazine was used in this study as a postive control and produced a dose-dependent inhibition of neutrophil degranulation caused by fMLP/CB.

fMLP/CB ($0{\cdot}3\,\mu M/5\,\mu g\,mL^{-1}$) or PMA (3 nM) stimulated superoxide anion formation ($1{\cdot}23 \pm 0{\cdot}12$ and $2{\cdot}58 \pm 0{\cdot}13$ nmol per 30 min per 10^6 cells, respectively) from rat neutrophils. As shown in

Table 3. ^{13}C NMR data for various chalcones and dihydrochalcones.

Compound	**1**[a]	**2**[b]	**3**[c]	**4**[c]	**5**[b]	**6**[b]	**7**[b]	**8**[b]	**9**[b]	**10**[b]	**11**[b]	**12**[b]	**13**[b]	**14**[b]
C-1′	121·4	119·9		123·7	119·8	119·7	119·7	119·6	129·5	128·0	129·6	128·1	118·9	118·9
C-2′	165·1	163·6	146·9	154·9	157·8	157·8	157·9	158·0	153·6	153·0	153·2	152·3	156·2	156·8
C-3′	117·8	118·7	133·6	116·6	117·8	117·5	119·0	119·4	114·4	115·1	115·2	115·2	119·2	119·5
C-4′	137·8	136·5	129·6	124·7	123·5	123·5	123·7	124·0	127·3	127·6	127·6	127·7	124·7	124·8
C-5′	119·6	118·9	135·4	153·6	151·6	151·6	151·7	151·8	152·6	152·2	152·3	153·2	147·5	147·3
C-6′	129·8	129·6		114·6	113·5	112·9	112·9	113·0	113·4	114·7	114·4	114·3	114·6	114·5
C-1	123·3	133·1	128·1	130·9	127·6	127·3	131·8	133·1	133·7	133·8	133·8	133·8	140·6	139·1
C-2	158·9	129·8	115·8	115·4	110·4	130·5	128·7	129·8	129·4	129·1	129·1	129·1	128·6	128·7
C-3	117·8	129·3	146·4	146·8	149·3	114·5	129·8	129·4	129·1	129·3	129·4	129·4	128·4	129·8
C-4	131·8	136·9	150·1	150·0	151·9	162·0	141·6	136·9	136·0	135·9	135·9	135·9	126·3	132·1
C-5	121·6	129·3	116·6	115·5	111·2	114·5	129·8	129·4	129·1	129·3	129·4	129·4	128·4	129·8
C-6	133·9	129·8	123·7	128·2	123·1	130·5	128·7	129·8	129·4	129·1	129·1	129·1	128·6	128·7
C-α	120·4	120·6	119·2	119·4	119·2	119·2	119·3	120·6	119·4	120·4	120·3	120·4	40·1	39·8
C-β	142·5	143·9	146·4	146·5	145·8	145·4	145·7	144·1	141·5	140·9	140·8	140·8	29·9	29·2
OCH_3				56·2; 56·8	56·2	55·4; 56·1	56·1	56·2	55·8; 56·5					
OCH_2										64·1; 65·2	70·2; 71·1	68·4; 69·2		
CH_2											22·6; 22·7	19·2; 19·4		
$CH_2CH_2CH_3$												31·4; 31·5		
C=O	196·0	193·5	184·3	195·1	193·2	193·0	193·4	193·1	192·0	191·8	191·8	191·9	204·8	204·8
CH_3							21·6			14·8; 15·0	10·5; 10·6	13·7; 13·8		

[a]Measured in $(CD_3)_2CO$. [b]Measured in $CDCl_3$. [c]Measured in CD_3OD.

Table 6, compounds **4**–**9** had potent inhibitory effects on fMLP/CB-induced superoxide anion formation from rat neutrophils, while **3**, **4** and **8** also showed potent inhibitory effects on the PMA-induced response. Compounds **10**–**12** (with two alkyl chains of C_2-C_4 at the 2′ and 5′ positions of **16**) and **9**–**12** (with two alkyl chains of C_1-C_4 at the 2′ and 5′ positions of **16**) did not inhibit the superoxide anion formation caused by fMLP/CB or PMA, respectively. These results indicate that the introduction of a lipophilic alkyl group at the 2′ and 5′ positions of **16** might attenuate its inhibitory effects on fMLP/CB- and PMA-induced responses. Reduction of **15** and **16** to **13** and **14**, respectively, did not inhibit fMLP/CB- or PMA-induced superoxide anion formation from rat neutrophils (Hsieh et al 1998), suggesting that the enone moiety of chalcones is also required for the inhibition of superoxide anion formation from rat neutrophils. Chalcones (except for **2**, **4**, **8**, and **10**–**14**) showed different potencies for the inhibition of fMLP/CB- and PMA-induced responses. It has been reported that fMLP and PMA induce superoxide anion formation by activating the same oxidase in neutrophils but that they utilize different transduction mechanisms and are regulated differently (Segal & Abo 1993). Our results indicated that the mechanisms by which compounds **1** and **3** inhibit superoxide anion formation were different from those of **5**–**7** and **9**. These inhibitory compounds exert better stimulant selectivity than trifluoperazine. Trifluoperazine was used in this study as a positive control and produced a dose-depen-

Table 4. The inhibitory effects of chalcones on the release of β-glucuronidase and histamine from rat peritoneal mast cells stimulated with compound 48/80.

Compound	IC50 (μM)[a]	
	β-Glucuronidase	Histamine
1	45·8 ± 7·0	54·1 ± 9·3
2	>30 (10·2 ± 5·0)	>30 (−0·1 ± 3·2)
3	>10 (13·9 ± 3·7)	>10 (35·5 ± 2·8)
4	>30 (23·0 ± 2·0)	>30 (30·6 ± 0·2)
5	65·3 ± 6·4	80·8 ± 17·2
6	81·0 ± 6·7	>100 (38·2 ± 7·9)
7	80·1 ± 11·9	>100 (47·6 ± 6·5)
8	>30 (16·7 ± 5·7)	>30 (5·1 ± 2·2)
9	88·8 ± 8·5	>100 (39·2 ± 5·9)
10	>100 (16·2 ± 2·5)	>100 (15·2 ± 3·5)
11	>100 (8·6 ± 8·2)	>100 (4·5 ± 3·0)
12	>100 (14·9 ± 7·9)	>100 (3·6 ± 3·7)
13	>100 (28·1 ± 1·5)	>100 (14·3 ± 0·5)
14	>30 (22·0 ± 6·4)	>30 (9·0 ± 4·2)
15	20·8 ± 0·84[b]	30·1 ± 2·96[b]
16	>100 (28·7 ± 3·7)[c]	>100 (41·7 ± 3·0)[c]
Mepacrine	21·2 ± 2·2	18·4 ± 2·5

Results are presented as average ± s.e.m. (n=3–5). [a]When 50% inhibition could not be reached at the highest concentration, the % of inhibition is given in parentheses. [b]Data from Lin et al (1997). [c]Data from Hsieh et al (1998).

Table 5. The inhibitory effects of chalcones on the release of β-glucuronidase and lysozyme from rat neutrophils stimulated with fMLP/CB.

Compound	IC50 (μM)[a]	
	β-Glucuronidase	Lysozyme
1	1·6 ± 0·2	1·4 ± 0·2
2	>30 (33·4 ± 4·0)	>30 (19·4 ± 2·4)
3	>10 (45·0 ± 11·8)	>10 (30·6 ± 12·2)
4	16·9 ± 2·8	13·1 ± 2·0
5	5·6 ± 1·4	7·3 ± 1·4
6	11·9 ± 1·4	16·9 ± 2·4
7	17·4 ± 1·8	7·9 ± 2·7
8	>30 (29·1 ± 5·1)	>30 (16·0 ± 1·4)
9	11·9 ± 1·4	14·7 ± 2·8
10	>100 (34·7 ± 3·9)	>100 (21·9 ± 9·1)
11	>100 (30·1 ± 4·4)	>100 (16·3 ± 3·4)
12	>100 (25·8 ± 4·3)	>100 (17·0 ± 2·9)
13	47·7 ± 7·7	53·4 ± 7·7
14	16·3 ± 0·9	>30 (37·2 ± 3·2)
15	2·3 ± 0·2[b]	3·6 ± 0·3[b]
16	0·6 ± 0·1[c]	2·6 ± 0·2[c]
Trifluoperazine	15·2 ± 2·6	13·8 ± 2·2

Results are presented as average ± s.e.m. (n=3–5). [a]When 50% inhibition could not be reached at the highest concentration, the % of inhibition is given in parentheses. [b]Data from Lin et al (1997). [c]Data from Hsieh et al (1998).

dent inhibition of superoxide anion formation from rat neutrophils with fMLP/CB and PMA, respectively.

Following the activation of macrophages, NO was generated in response to LPS (Ding et al 1988). As shown in Table 7, compound **13** showed significant and concentration-dependent inhibitory effects on NO accumulation from Raw 264·7 mouse macrophage-like cells (70·5 ± 1·6 μM nitrite accumulation as control value). LPS also activated murine microglial cell lines N9 to produce NO (Meda et al 1995). Compounds **9**–**12** showed potent and concentration-dependent inhibition of LPS-induced NO formation from N9 cells (14·7 ± 0·6 μM nitrite accumulation as control value), indicating that compounds with two alkyl chains of C_2-C_4 at the 2′ and 5′ position of **16** exhibited stronger inhibitory effects on NO formation from N9 cells. This shows that increasing the lipophicity of a compound enhances its inhibitory effects on NO formation from N9 cells. Compound **13** (reduction from **15**) exerted significant inhibitory effects on NO formation from Raw 264·7 cells stimulated with LPS, while the effects of **14** (reduction from **16**) were insignificant. N^G-Nitro-L-arginine methylester (L-NAME) is a competitive inhibitor of NO synthase (Crossin 1991), and hence inhibited the NO formation from neutrophils.

Our previous report indicates that the anti-inflammatory effect of **16** is not due to the release of steroid hormones from the adrenal gland but to the suppression of chemical mediators released from mast cells and neutrophils (Hsieh et al 1998). The present study has also demonstrated that most of the chalcone derivatives studied have inhibitory effects on inflammatory cells in-vitro. The NO formation from N9 cells was inhibited by com-

Table 6. The inhibitory effects of chalcones on superoxide anion formation from rat neutrophils stimulated with fMLP/CB or PMA.

Compound	IC50 (μM)[a]	
	fMLP/CB	PMA
1	>100 (43·0 ± 10·5)	46·6 ± 7·4
2	>30 (28·7 ± 7·9)	>30 (11·8 ± 9·4)
3	>100 (15·4 ± 8·6)	3·9 ± 0·4
4	6·2 ± 3·3	4·9 ± 0·8
5	2·8 ± 0·2	>100 (33·4 ± 5·5)
6	35·5 ± 10·1	>100 (−34·3 ± 9·9)
7	27·7 ± 4·9	>100 (−78·9 ± 21·7)
8	4·2 ± 0·5	4·2 ± 0·6
9	6·3 ± 0·5	>100 (39·9 ± 7·6)
10	>100 (29·8 ± 4·5)	>100 (2·1 ± 7·2)
11	>100 (7·3 ± 8·2)	>100 (15·4 ± 11·9)
12	>100 (27·6 ± 9·0)	>100 (16·1 ± 6·4)
13	>10 (10·7 ± 8·9)	>10 (34·8 ± 7·2)
14	>3 (−26·7 ± 6·4)	>3 (45·3 ± 7·7)
15	2·6 ± 0·1[b]	–
16	>100 (19·5 ± 1·7)[c]	>100 (40·3 ± 14·4)[c]
Trifluoperazine	8·0 ± 0·2	5·0 ± 0·3

Results are presented as average ± s.e.m. (n=3–5). [a]When 50% inhibition could not be reached at the highest concentration, the % of inhibition is given in parentheses. [b]Data from Lin et al (1997). [c]Data from Hsieh et al (1998).

Table 7. The inhibitory effects of chalcones on the accumulation of NO_2^- from Raw 264·7 cells stimulated with LPS and N9 cells stimulated with LPS.

Compound	IC50 (μM)[a]	
	Raw 264·7 cells	N9 Cells
1	–	–
2	>30 (25·9 ± 1·7)	>30 (−34·3 ± 2·3)
3	–	–
4	–	–
5	–	–
6	–	–
7	–	–
8	>30 (28·2 ± 5·7)	>30 (−6·5 ± 0·6)
9	>10 (8·0 ± 0·8)	5·8 ± 0·2
10	>3 (14·9 ± 0·4)	1·0 ± 0·1
11	>3 (17·5 ± 0·7)	0·7 ± 0·06
12	>10 (29·7 ± 1·9)	1·9 ± 0·1
13	11·8 ± 0·9	>3 (48·8 ± 3·3)
14	>1 (12·4 ± 1·1)	>3 (5·9 ± 1·5)
L-NAME	1·1 ± 0·1	0·3 ± 0·01

Results are presented as average ± s.e.m. (n=3–5). [a]When 50% inhibition could not be reached at the highest concentration, the % of inhibition is given in parentheses. –, Not determined.

pound **9**–**12**; whether this may have beneficial effects on central neurodegenerative disorders needs further investigation.

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