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Anti-inflammatory Profile of *N*-Phenylpyrazole Arylhydrazone Derivatives in Rats

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Abstract

A series of synthetic *N*-phenylpyrazole arylhydrazone compounds, rationally designed as mixed-hybrid isosteres of two known inhibitors of prostaglandin synthase and 5-lipoxygenase enzymes, BW-755c and CBS-1108, has been investigated for anti-inflammatory activity in the carrageenan-induced pleurisy model in rats. The compounds have different oxygenated substituent groups in the aryl group of the hydrazone framework to ensure a different range of redox properties. A new arylhydrazone derivative, 2,6-di-*tert*-butyl-4-(4-nitro-3-methyl-*N*-phenylpyrazol-5-yl-hydrazonomethyl)phenol, was also synthesized and tested for anti-inflammatory activity.

Although all the compounds significantly inhibited (by 30–90%) neutrophil accumulation in the pleural cavity, there was great variability in the anti-oedematogenic effect of the compounds (3–96%). 5-(4′-Hydroxy-3′-methoxybenzylidene)hydrazone-3-methyl-4-nitrophenylpyrazole was the most active compound in this series; it had a remarkable anti-inflammatory profile, almost blocking both assays. In contrast, the compound with a 2,6-di-*tert*-butylated hydroxybenzene ring on the hydrazone group inhibited neutrophil migration only.

These results will be useful for further structure–activity relationship studies devoted to improving the dual prostaglandin synthase-5-lipoxygenase activity of these derivatives and determining the minimum structural requirements necessary for this activity.

A substantial body of evidence suggests the involvement of the products of the arachidonic acid metabolic pathway (eicosanoids) as mediators of a variety of cellular functions (Davies et al 1984; Henderson 1994; Vane & Botting 1995). Excessive production of arachidonic acid metabolites has been shown to be important in the pathophysiology of thrombosis, inflammation, asthma and allergy (Heller et al 1998). The eicosanoids synthesized during the acute inflammatory response are responsible for several characteristic effects including vasodilatation, increases in vascular permeability and leukocyte migration to the injured site (Vinegar et al 1982).

The therapeutic mode of action of classical non-steroidal anti-inflammatory drugs (NSAIDs), such as acetylsalicylic acid, indomethacin, diclofenac and meloxican is primarily explained by their inhibitory effect on prostaglandin synthase, the key enzyme of the prostaglandin pathway (Vane & Botting 1995). Leukotrienes, the products of the 5-lipoxygenase pathway in the arachidonic acid cascade, have been implicated in many events in the development of pulmonary inflammation (Batt 1992; Thien & Walter 1995) and are also an important therapeutic target (Engels & Nijkamp 1998). Thus, it is conceivable to develop dual inhibitors of prostaglandin synthase and 5-lipoxygenase as potential therapeutic agents in allergic and inflammatory diseases.

In earlier studies we have described the synthesis, and the analgesic (Matheus et al 1991) and anti-platelet activity, of an analogous series of *N*-phenylpyrazole arylhydrazone derivatives (Silveira et al 1993). These compounds were designed rationally

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by molecular hybridization of two known inhibitors of prostaglandin synthase and 5-lipoxygenase—the hydrazones BW-755c (Higgs et al 1980) and CBS-1108 (Sincholle et al 1985; Ghiglieri-Bertez et al 1987) as a new class of mixed-hybrid isosteres (Figure 1). Considering that most 5-lipoxygenase inhibitors have specific redox properties (Musser & Krieft 1992; Müller et al 1998), the series of hydrazone derivatives described herein (Figure 1), were also designed to have, in the aryl group of the hydrazone framework, different functional substituent groups with iron-binding capacity, for example catechol, monomethylated catechol and other aryl-oxygenated sub-units, structurally related but with different redox properties (Flynn et al 1991; Müller et al 1998). In designing this series of hydrazone derivatives we also planned to introduce a 2,6-di-*tert*-butylated phenol residue into the hydrazone framework because of the particular redox and 5-lipoxygenase inhibitory properties attributed to this framework (Lazer et al 1989, 1990; Wong et al 1992; Mullican et al 1993; Unangst et al 1994; Cuadro et al 1998). In addition, we considered the isosteric relationship between *ortho*-nitro and *ortho*-amino at C-4 and C-5 of the pyrazole ring of these derivatives (**1**–**11**) and the *ortho*-carbohydroxyl or *ortho*-amino group present in some active NSAIDs (Freitas 1991), to mimic the anthranilic acid function of the arylhydrazones. Furthermore, because the imine double bond in these hydrazone derivatives can be regarded as an azo-styryl group, which is structurally related to known dual inhibitors belonging to an active class of styryl derivatives (Flynn et al 1991), this structural similarity was also considered attractive in the study of the anti-inflammatory profile of these compounds. Thus, the principal aim of this work was to investigate the anti-inflammatory effects of this series of hydrazone derivatives (**1**–**11**). For this, we used rat acute pleurisy as an inflammatory model in which the participation of prostaglandins and leukotrienes as mediators in the development of pleural oedema and neutrophil infiltration has been characterized (Vinegar et al 1982).

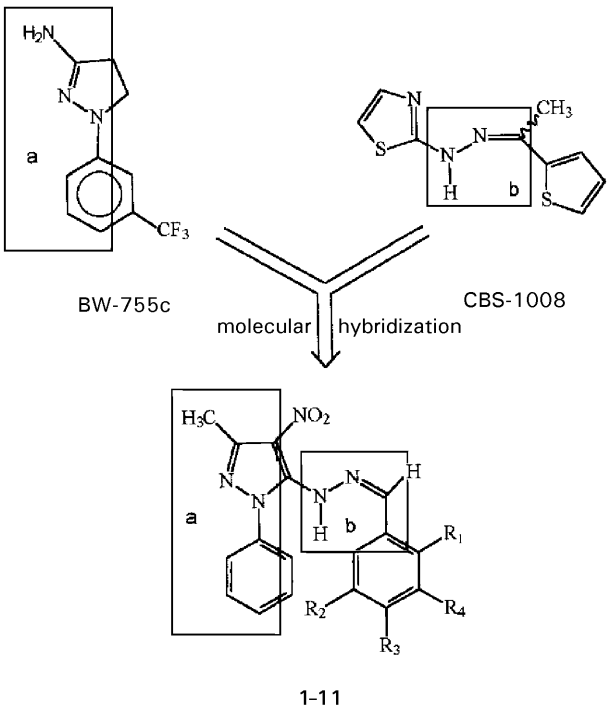

Figure 1. Rational basis for the structural design of *N*-phenylpyrazole arylhydrazone derivatives **1**–**11**. $R_4 = H$ in compounds **1**–**10**. **1**, R_1, R_2, $R_3 = H$; **2**, $R_1 = OH$, R_2, $R_3 = H$; **3**, $R_1 = H$, R_2, $R_3 = OH$; **4**, R_1, $R_2 = H$, $R_3 = OH$; **5**, $R_1 = H$, $R_2 = OCH_3$, $R_3 = OH$; **6**, $R_1 = H$, $R_2 = OH$, $R_3 = OCH_3$; **7**, $R_1 = H$, R_2, $R_3 = OCH_3$; **8**, $R_1 = H$, R_2, $R_3 = CH_2OCH_2$; **9**, $R_1 = NO_2$, R_2, $R_3 = CH_2OCH_2$; **10**, $R_1 = Br$, R_2, $R_3 = CH_2OCH_2$; **11**, $R_1 = H$, $R_4 =$ *tert*-butyl; $R_3 = OH$.

Materials and Methods

Chemistry

Melting points (mp) were determined with a Thomas-Hoover apparatus. Proton magnetic resonance (^{1}H NMR) was determined, in deuterated chloroform containing 1% (approx.) tetramethylsilane as internal standard, by means of 200 MHz Bruker AC 200 spectrometers. Splitting patterns are: s, singlet; br, broad; m, multiplet. Carbon magnetic resonance (^{13}C NMR) was determined with the same apparatus at 50 MHz. Mass spectra (MS) were obtained by electron impact (70 eV) with a GC/VG Micromass 12 spectrometer.

The progress of the reaction was monitored by thin-layer chromatography on 2·0 cm × 6·0 cm aluminium-backed plates precoated with 0·25-mm layers of silica gel 60 (HF-254; Merck). The developed chromatograms were visualized with ultraviolet light at 254 nm. Merck silica gel (70–230 mesh) was used for column chromatography. The solvents used were purified by standard procedures.

2,6-Di-tert *-butyl-4-(4-nitro-3-methyl*-N *-phenyl-pyrazol-5-yl-hydrazonomethyl)phenol (***11**)

A mixture of *N*-phenyl-3-methyl-4-nitro-5-hydrazinepyrazole (**12**; 0·233 g, 1 mmol) (Khan & Freitas 1983) and 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde (**13**; 0·234 g, 1 mmol) in absolute ethanol (10 mL) was stirred under reflux with a catalytic amount of hydrochloric acid as catalyst for 2 h (approx.) The title compound was isolated by concentration of the reaction mixture under

reduced pressure, then neutralization with 10% aqueous sodium bicarbonate. The resulting precipitate was removed by filtration, washed with water and dried in air to give a yellow amorphous solid (**11**; 0·394 g, 85%), mp 223–225°C. IR (KBr) cm^{-1}: 3616, 3420, 3278, 1612, 1535, 1436, 1325; ^1H NMR (200 MHz, $CDCl_3$) δ: 10·18 (br, 1 H, NH) (1 H, s), 7·8 (s, 1 H, CH = N), 7·36–7·48 (m, 5 H), 6·98 (s, 2 H), 5·46 (s, 1 H, OH), 2·57 (s, 3 H, CH_3), 1·34 ppm (s, 18 H *tert*-butyl); ^{13}C NMR (50 MHz, $CDCl_3$) δ: 14·31 (CH_3), 140·04 (C-3), 124·22 (C-4), 146·11 (C-5), 147·59 (HC = N), 142·61 (C-1′), 128·46 (C-2′, C-6′), 136·25 (C-3′, C-5′), 156·06 (C-4′), 34·16 ($-C(CH_3)_3$), 30·08 ppm ($-C(CH_3)_3$); MS m/z (%): M^+ 449 (100), 232 (6), 77 (38).

Animals and treatment

Male Wistar rats, 150–180 g, supplied by the breeding facilities of the Oswaldo Cruz Foundation (Rio de Janeiro, Brazil), were housed in temperature-controlled rooms and had free access to water and food until use. During all experiments the animals were treated in accordance with published regulations for animal experiments.

Hydrazone compounds **1**–**11** at concentrations of 36–42 μM in propylene glycol were administered subcutaneously 1 h before induction of pleurisy. In some experiments three groups of animals were pretreated with known anti-inflammatory drugs (indomethacin (8·5 μM); nordihydroguaiaretic acid (330 mM) and phenidone (615 mM)) using the same protocol.

Induction of acute pleurisy

Acute pleurisy was induced as described elsewhere (Utsunomiya et al 1994). Briefly, carrageenan (Type I, Sigma; 500 μg in a final volume of 100 μL) was injected into the thoracic cavity of normal or pretreated rats. In the control group carrageenan was replaced by sterile saline. Rats were killed by ether anaesthesia 4 h after carrageenan injection. The thoracic cavities were opened and rinsed with phosphate-buffered saline (PBS; 3 mL) containing heparin (10 int. units mL^{-1}). The pleural wash was collected and its volume measured with a graduated plastic syringe. The exudate volume (mL) was obtained by subtracting the volume of PBS-heparin (3 mL) from the total volume of fluid collected.

The total number of leukocytes in the pleural fluid was determined in Neubauer chambers after dilution with Türk solution. Differential analysis of leukocytes was performed by microscopic counting of May-Grünwald-Giemsa-stained slides. Results were expressed as millions of cells/cavity.

Statistical analysis

Results are presented as means ± s.d. from at least six animals. The data were analysed by analysis of variance then Bonferroni's *t*-test; P values $\leq 0{\cdot}05$ were considered to be indicative of significance. Occasionally results were also shown as percentage inhibition relative to controls (untreated animals).

Results and Discussion

The synthesis of known hydrazone derivatives **1**–**10** was performed by classical methods as described previously (Freitas et al 1995; Todeschini et al 1996). The new derivative, **11**, containing the 2,6-di-*tert*-butylphenol group, was prepared in high yield by a synthetic method described elsewhere (Freitas 1991), starting from *N*-phenyl-3-methyl-4-nitro-5-hydrazinepyrazole, **12**, by acid-catalysed condensation with 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde, **13** (Figure 2). The diastereomeric nature of the nitrogen-carbon double bond in this new compound (**11**) was established by ^1H NMR and is in agreement with previous reports from this laboratory (Gaston et al 1996; Todeschini et al 1998) and elsewhere (Easmon et al 1997). For instance, the singlet signal for $-CH=N-$ appears in the ^1H NMR spectra at $\delta = 8{\cdot}32$ ppm, with a chemical shift typical of the *Z* configuration, reflecting a diastereoselective condensation step in the construction of the hydrazone double bond. In addition, infrared spectral data indicate that in this hydrazone the azo form seems not to be involved as a possible tautomer (Gaston et al 1996; Todeschini et al 1998).

To investigate the effect of these structurally related arylhydrazone derivatives (**1**–**11**) on the acute pleurisy induced by carrageenan, the compounds were administered subcutaneously, at concentrations of 36 to 42 μM, 1 h before induction of acute pleurisy with carrageenan. After 4 h the volume of exudate and the number of neutrophils accumulated in the pleural cavities were measured (Table 1). The data shown in Table 1 indicate that all compounds tested, almost at the same concentration, significantly inhibited, from 30 to 90%, neutrophil accumulation in the rat pleural cavities.

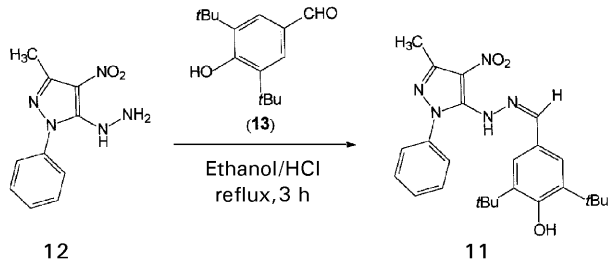

Figure 2. The synthesis of 2,6-di-*tert*-butyl-4-(4-nitro-3-methyl-*N*-phenylpyrazol-5-yl-hydrazonomethyl)phenol, **11**.

However, there was substantial variability (from 3 to 96%) among the inhibitory effects of these hydrazone compounds on exudate formation induced by carrageenan injection. Interestingly, compound **1**, which has no substituent groups on the aryl ring, had remarkable anti-inflammatory effect, inhibiting the neutrophil migration (66%) and plasma exudation (40%). The presence of a hydroxyl group in the *ortho* position (R_1) of the aryl ring, as in compound **2**, seems to be needed for inhibition of neutrophil migration but has a deleterious effect on plasma exudation activity (Table 1). Among all the compounds tested for anti-inflammatory activity the most potent was arylhydrazone **5**; 40 μM almost completely blocked oedema formation and neutrophil accumulation (approx. 95%) (Table 1). At a concentration of 40 μM compound **5** had a greater effect than nordihydroguaiaretic acid and phenidone (two known 5-lipoxygenase inhibitors) at higher concentrations (data not shown). In contrast, compound **6**, the regioisomer of compound **5**, at the same concentration, although having a similar effect on inhibition of cell migration (83%), reduced the accumulation of exudate induced by carrageenan by 33% only. Nevertheless, investigation of structure–activity relationships in this series of compounds is not simple because compounds **4** (42 μM) and **7** (39 μM), with an oxygenation pattern in the aryl ring different from that in **5**, not only reduced the anti-migratory effect, but also abolished the anti-oedematogenic effect. These results seem to indicate that the presence of a hydroxyl group in the *meta* position (R_2) of the aryl ring is not essential for potent anti-oedematogenic activity of the arylhydrazone derivatives (Table 1). For example, compound **3** (42 μM) had a weak inhibitory effect on pleural exudate formation (22% inhibition) and was less potent than **5** at inhibiting neutrophil migration (42%). The introduction of a methylenedioxy group in the aryl ring, as seen for **8**, resulted in strong inhibition at a concentration of 41 μM, similar to that of **5** at 40 μM, of the neutrophil migration induced by carrageenan, but with a significant decrease in anti-oedematogenic effect (38%). Substitution in the *ortho* position of the aryl ring in **8** significantly changed the anti-inflammatory activity, and the presence of an *ortho*-nitro group, as in compound **9**, increased the anti-oedematogenic effect to 60% (approx.) with modest reduction of the inhibition of cell accumulation. The presence of a bromine atom in the *ortho* position of the aryl ring containing the methylenedioxy group, as in compound **10**, reduced the anti-migratory activity without altering the effect on exudate formation (Table 1). Finally, at a concentration of 40 μM the new synthetic compound (**11**) with the 2,6-di-*tert*-butylphenol group in the aryl ring, inhibited neutrophil migration by 70% (approx.) but did not significantly affect the oedematogenic effect of carrageenan injection (Table 1). Curiously, compound **11**, which has particular redox properties, had ''redox-type'' inhibitory behaviour, possibly because of its potential antioxidant activity. It can be concluded that the series of arylhydrazone derivatives (**1**–**11**) described here, with minor structural modifications in the aryl ring of the hydrazone group, can be regarded as a new series of anti-inflammatory compounds in which the presence of different oxygenated substituents with distinct redox properties ensure the expected anti-inflammatory profile.

These results will be very useful for further studies of structure–activity relationships, in progress in our laboratory, to improve the dual prostaglandin synthase-5-lipoxygenase activity of these derivatives and to determine the minimum structural requirements necessary for this activity.

Table 1. Effect of *N*-phenylpyrazole arylhydrazone compounds **1**–**11** on the pleurisy induced by carrageenan.

Compound	Concn (μM)	Neutrophils $\times 10^6$/cavity	Inhibition (%)	Exudation volume (μL)	Inhibition (%)
None	–	38·6 ± 3·9	–	1·2 ± 0·2	–
1	40	10·2 ± 1·4	66·0	0·61 ± 0·2	40·3
2	40	5·8 ± 0·56	78·7	0·77 ± 0·2	28·0
3	42	22·2 ± 1·4	42·4	1·12 ± 0·4	21·6
4	42	26·5 ± 1·0	31·2	1·08 ± 0·2	10·0
5	40	2·5 ± 0·9	93·4	0·04 ± 0·01	96·6
6	40	6·6 ± 0·8	83·0	0·80 ± 0·2	33·3
7	39	17·8 ± 2·73	53·8	1·16 ± 0·3	3·3
8	41	2·7 ± 0·6	92·9	0·75 ± 0·2	37·5
9	36	7·9 ± 1·7	80·0	0·48 ± 0·1	60
10	40	13·1 ± 1·6	73·7	0·79 ± 0·1	40·8
11	40	12·13 ± 2·3	70·1	1·02 ± 0·1	9·6

The animals were pretreated subcutaneously with the indicated concentration of each hydrazone 1 h before intrathoracic injection of carrageenan (500 μg). Neutrophil migration and exudate volume into the pleural cavities were evaluated 4 h later. Results are also shown as percentage inhibition compared with the control groups (no pretreatment).

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