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New Pyrazolylhydrazone Derivatives as Inhibitors of Platelet Aggregation

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Abstract—A series of 5-pyrazolylhydrazone derivatives was designed to be mixed hybrid isosteres of both BW755C and CBS-1108, which belong to the class of dual cyclo-oxygenase and 5-lipoxygenase inhibitors. Some derivatives of this series inhibit the in-vitro platelet aggregation of citrated platelet-rich rabbit plasma induced by ADP (5 μM), collagen (5 μg mL^{-1}) and arachidonic acid (100 μM). The structure–activity relationships of this class of compounds were determined from these results. When ADP is used as the aggregation inducer, the presence of free oxygenated substituents at the *p*-position in the phenyl subunit of the hydrazone moiety favours inhibitory activity; *p*-methoxyformylbenzene-5-(1-phenyl-3-methyl-4-nitropyrazolyl)hydrazone (100 μM), which has a methoxy group at this position was the most active with 62·8% inhibition of aggregation. In contrast, substitution in the aryl ring does not affect the aggregation induced by collagen, whereas the non-substituted compound, formylbenzene-5-(1-phenyl-3-methyl-4-nitropyrazolyl)hydrazone, showed similar activity to those of substituted derivatives. In the arachidonic acid assays, the presence of an aryl ring linked to the hydrazone moiety, with an adequate electronic density at the ring due to the nature of its substitutents, is an important structural requirement for inhibitory activity.

Compounds such as BW755C and CBS-1108 have a wider range of anti-inflammatory activities than classical non-steroidal anti-inflammatory drugs like aspirin and indomethacin. Based on dual inhibition of cyclo-oxygenase and 5-lipoxygenase, these compounds reduce prostaglandin concentration and leucocyte migration in inflammatory exudates (Higgs et al 1980; Sincholle et al 1985). However, other studies have shown that BW755C has cytotoxic and haemolytic activity (Ahnfelt-Ronne & Arrigoni-Martelli 1982; Vane 1987).

Searching for new compounds which have potential inhibitory activity on the arachidonic acid cascade, a series of 5-pyrazolylhydrazone derivatives was synthesized from the appropriate pyrazolylhydrazines and the corresponding aldehydes (Freitas et al 1987).

The general structure of derivatives (Table 1) was designed based on the bioisosterism concept represented by the presence of an endocyclic aryl hydrazone moiety in BW755C, that corresponds to that represented by the *N*-arylpyrazol ring and the presence of the same structural subunit in CBS-1108 as a spacer group between thiazolyl and thienyl rings.

Some of the compounds in the present series had been previously evaluated pharmacologically for anti-oedemic and analgesic activities (Pereira 1989). Compound 1 (Table 1) was the most active compound as an anti-oedemic agent, with a higher potency than indomethacin in the modified carrageenan-induced rat-paw oedema test (Winter et al 1962).

Further studies demonstrated that some derivatives inhibit ADP-, collagen- and arachidonic acid-induced in-vitro platelet aggregation in platelet-rich plasma from rabbits (Silveira et al 1991).

In the present study we used 12 compounds (Table 1), among them nine new derivatives, in order to obtain a better correlation between structure and platelet anti-aggregating activity for this series.

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Materials and Methods

Isolation of rabbit platelets

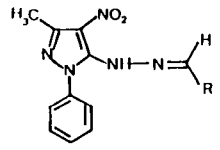
Blood samples were collected by heart puncture into a plastic flask containing 3·8% tri-sodium citrate (1:9 v/v) from rabbits weighing 2·5–3·0 kg. Platelet-rich plasma (PRP) was prepared by centrifugation (500 *g* for 10 min) at room temperature (21°C) and the platelet count was adjusted to 5×10^8 platelets mL^{-1}. Each PRP sample was used within 3 h of its collection.

Platelet aggregation

Rabbit PRP (400 μL) was incubated at 37°C for 1 min in a BPI–Tecnologia (MAP 002) aggregometer (Born & Cross 1963) with continuous stirring at 900 rev min^{-1} and then stimulated with ADP (5 μM in distilled water), collagen (5 μg mL^{-1} in 0·9% NaCl) or arachidonic acid (100 μM in ethanol). Collagen was prepared by the method of Cazenave et al (1983).

The changes in light transmission were recorded for 3 min after stimulation with these agents. Test compounds and the solvent used in dissolution of the pyrazolic compounds (0·5% DMSO; 2 μL in volume) (Gerrard 1982) were added to the platelet suspension 5 min before addition of the aggregating agent. The compounds were studied at 1, 10 and 100 μM with the exception of compounds 8, 9 and 10 which were not soluble at 100 μM. Indomethacin, a classical cyclo-oxygenase

Table 1. Structure of 5-pyrazolylhydrazone derivatives.



Compound	Mol. wt	mp (°C)	R
1	416	220–1	3′,4′-Methylenedioxy-6-nitro-phenyl
2	311	178–9	2-Furyl
3	337	197–8	2-Furyl-acriloyl
4	351	210–1	4′-Methoxy-phenyl
5	367	224–5	4′-Hydroxy-3′-methoxy-phenyl
6	365	193–4	3′-Hydroxy-phenyl
7	367	226–7	3′Hydroxy-4′-methoxy-phenyl
8	381	222–3	3′,4′-Di-methoxy-phenyl
9	359	231–2	3′,4′-Di-hydroxy-phenyl
10	365	228–9	3′,4′-Methylenedioxy-phenyl
11	321	205–6	Phenyl
12	337	198–9	2′-Hydroxy-phenyl

inhibitor (Vane 1971), was used as a standard (10 μM, Tris–HCl buffer, pH 7·4) under the same conditions as described above. The concentrations of the aggregation inducers and the tested substances are reported as final concentrations in PRP.

The platelet aggregation is expressed as percentage of platelet aggregation for ADP and arachidonic acid, and as the maximum rate of aggregation (slope) for collagen (Cazenave et al 1983).

Materials

Adenosine 5-diphosphate (ADP), arachidonic acid and collagen bovine type I were obtained from Sigma St Louis, MO. Indomethacin was obtained from Merck, Sharp & Dohme São Paulo, Brazil.

Statistical analysis

The results are expressed as the mean ± s.e.m. for n experiments in triplicate. Student's paired *t*-test was used for the indomethacin and DMSO assays. Analysis of variance (one-way, Scheffé test) was used for the experiments with pyrazolic compounds. A P value of <0.05 was taken as significant in both cases.

Results

The results obtained for 5-pyrazolylhydrazones are presented in Table 2, with the exception of compounds 6, 8, 9 and 10 which did not show any significant activity on platelet aggregation induced by the three agonists.

Effect of pyrazolylhydrazones on ADP-induced platelet aggregation

Table 2 shows that compound 4 (100 μM) was the most active with 62·8% of the inhibition of aggregation followed by compound 3 with the rate of 58·3% in the same concentration. Compound 1 inhibited 48·7% of aggregation while compound 12, at 100 μM, demonstrated a lower but significantly different activity (41·7%; $P<0.05$). It is important to note that compound 12 showed a similar inhibitory activity

Table 2. Effect of 5-pyrazolylhydrazone derivatives on in-vitro platelet aggregation of rabbit citrated platelet-rich plasma induced by ADP, collagen and arachidonic acid.

		ADP (5 μM)			Collagen (5 μg mL^{-1})			Arachidonic acid (100 μM)	
Compounds	Concentration (μM)	n	Aggregation (%)	Inhibition (%)	n	Aggregation (slope)	Inhibition (%)	n	Aggregation (%)	Inhibition (%)
	0	37	56·1 ± 0·8		60	17·3 ± 0·4		56	80·3 ± 0·9	
1	1	5	44·4 ± 2·9	20·9	5	13·7 ± 1·0	20·8	5	76·6 ± 6·3	4·6
	10	4	39·3 ± 2·8	30·0	4	11·1 ± 0·3	35·8	5	73·9 ± 5·1	8·0
	100	3	28·8 ± 1·2	48·7*	4	0·0 ± 0·0	100·0*	4	5·2 ± 2·8	93·5*
2	1	4	48·7 ± 1·9	13·2	7	14·0 ± 0·9	19·1	6	80·1 ± 2·9	0·3
	10	4	47·0 ± 2·6	16·2	4	11·2 ± 0·3	35·3	4	80·3 ± 4·0	0·0
	100	4	38·0 ± 1·8	32·3	6	0·2 ± 0·2	98·8*	5	0·9 ± 0·9	98·9*
3	1	5	44·5 ± 3·1	20·7	7	16·1 ± 1·3	6·9	4	85·0 ± 2·3	−5·9
	10	5	41·2 ± 2·6	26·6	6	15·1 ± 1·7	12·7	3	85·9 ± 2·0	−7·0
	100	5	23·4 ± 3·1	58·3*	5	2·6 ± 1·5	85·0*	3	70·0 ± 3·2	12·8
4	1	4	48·5 ± 2·7	13·6	3	17·8 ± 2·0	−2·9	5	90·6 ± 1·9	−12·8
	10	4	46·2 ± 0·3	17·7	4	13·9 ± 2·1	19·7	5	89·1 ± 2·6	−11·0
	100	4	20·9 ± 1·3	62·8*	4	0·7 ± 0·5	96·0*	5	77·8 ± 2·1	3·1
5	1	5	42·3 ± 1·9	24·6	7	13·0 ± 0·9	24·9	5	82·5 ± 4·5	−2·7
	10	5	41·2 ± 1·5	26·6	7	12·0 ± 0·3	30·6	5	80·9 ± 4·5	−0·8
	100	5	37·8 ± 1·2	32·6	7	4·1 ± 1·0	76·3*	4	71·4 ± 3·4	11·1
7	1	8	47·3 ± 1·8	15·7	6	12·8 ± 0·6	26·0	4	77·3 ± 3·8	3·7
	10	8	43·6 ± 1·7	22·3	6	12·4 ± 0·6	28·3	4	75·2 ± 5·3	6·4
	100	6	42·3 ± 1·5	24·6	6	4·0 ± 1·2	76·9*	3	67·3 ± 1·6	16·2
11	1	6	47·6 ± 3·3	15·2	8	13·6 ± 0·6	21·4	5	75·6 ± 4·0	5·9
	10	6	48·2 ± 3·6	14·1	4	13·7 ± 0·9	20·8	5	70·3 ± 2·7	12·5
	100	4	35·6 ± 7·5	36·5*	5	2·9 ± 1·4	83·2*	4	33·2 ± 3·8	58·7*
12	1	4	43·1 ± 2·8	23·2	5	14·2 ± 1·4	17·9	5	78·6 ± 4·0	2·1
	10	4	40·8 ± 2·9	27·3	5	12·6 ± 1·6	27·2	5	76·9 ± 4·4	4·2
	100	4	32·7 ± 3·0	41·7*	5	4·9 ± 2·7	71·7*	4	55·7 ± 2·5	30·6

n = number of experiments in triplicate; *$P<0.05$.

to compound 11 (100 μM; 36·5% of inhibition; $P<0·05$), in spite of the difference in the substitution pattern at the aromatic ring of the hydrazone unit.

DMSO showed neither pro-aggregating activity (n = 5; data not shown), nor did it significantly modify the aggregation induced by ADP (from 56·8 ± 2·4 to 48·5 ± 2·1; n = 3; $P>0·05$). Similarly, indomethacin did not modify the control value of platelet aggregation obtained with ADP (from 57·6 ± 2·5 to 56·9 ± 2·0; n = 3; $P>0·05$).

Effect of pyrazolylhydrazones on collagen-induced platelet aggregation

With the exception of compounds 6, 8, 9 and 10, all compounds at 100 μM exhibited rates of inhibition > 70% on collagen-induced platelet aggregation (Table 2).

DMSO significantly reduced the rate of platelet aggregation induced by collagen, by 17·7% (from 17·0 ± 0·5 to 14·0 ± 0·6; n = 8; $P<0·05$).

Indomethacin inhibited 94·6% of platelet aggregation obtained with collagen (from 18·5 ± 0·5 to 1·0 ± 0·5; n = 8; $P<0·05$).

Effect of pyrazolylhydrazones on arachidonic acid-induced platelet aggregation

Compounds 2, 1 and 11, at 100 μM, were the most active compounds on the platelet aggregation induced by arachidonic acid, with 98·9, 93·5 and 58·7% of inhibition, respectively (Table 2). On the other hand, these compounds at 1 and 10 μM did not demonstrate any activity.

However, compound 2, at 21·5 and 46·4 μM, inhibited in a concentration-dependent manner, the response induced by arachidonic acid. In contrast, higher concentrations of agonist reduced this inhibitory activity (n = 3; data not shown).

DMSO did not modify the platelet aggregation induced by arachidonic acid in a significant manner (from 80·3 ± 3·1 to 78·4 ± 3·2; n = 5; $P>0·05$).

Indomethacin totally inhibited the platelet aggregation induced by arachidonic acid (from 74·6 ± 1·4 to 0·0 ± 0·0; n = 4; $P<0·05$).

Discussion

The results of this study show that the 5-pyrazolylhydrazone derivatives, designed to have similar mixed structural features of both BW755C (Higgs et al 1979) and CBS-1108 (Sincholle et al 1985), are active inhibitors of ADP-, collagen- and arachidonic acid-induced in-vitro platelet aggregation in rabbit PRP.

DMSO, used as a vehicle for the pyrazolic compounds, as expected did not interfere with ADP- or arachidonic acid-induced platelet aggregation. In contrast, DMSO showed some inhibitory activity against collagen-induced platelet aggregation, albeit of minor relevance towards explaining the high inhibitory activity observed with the compounds.

Indomethacin does not interfere with ADP-induced platelet aggregation but inhibits the aggregation induced by collagen and arachidonic acid. Similar results had been obtained previously (Lewis & Watts 1982; Hwang 1985).

ADP-induced platelet aggregation in rabbits is obtained by interaction of an agonist with platelet membrane receptors, without interference of the release reaction and thromboxane A_2 synthesis (Cazenave et al 1979; Kinlough-Rathbone et al 1983).

In the present investigation, compound 1, which has a substituent at the *o*-position of the aryl ring, showed greater activity than compound 12 ($P<0·05$), which is monosubstituted with a hydroxyl group in the same position. These results could be related to the presence of different substituents in both compounds, showing opposite electronic effects (NO_2, electron-withdrawing; OH, electron-donating).

The monosubstitution pattern of the aryl ring when substituted by a methoxy, group at the *p*-position, as in compound 4, seems to confer a higher percentage of inhibition on platelet aggregation induced by ADP when compared with the unsubstituted derivative (compound 11), or with compounds having a *m*- and *o*-substituent hydroxy group (compounds 6 and 12, respectively) at 100 μM.

In fact, the *p*-oxygenated function belonging to a methylenedioxy bridge present in compound 10, seems to abolish the inhibitory activity, probably because of the substituent's rigid nature.

The inhibitory activity demonstrated by compounds 4, 3, 1, 12 and 11 on ADP-induced platelet aggregation was not expected. These results suggest that the compounds exhibit other properties which this study does not allow us to identify, as for example a possible interference in the interaction of ADP with the platelet membrane receptor. In contrast, since the compounds do not inhibit the ADP-induced shape change, they may not participate in the exposure process of fibrinogen receptors (Winocour et al 1981).

Collagen-induced platelet aggregation is mediated by thromboxane A_2 synthesis and by the release reaction from dense granules in rabbits (Charo et al 1977; Lewis & Watts 1982; Hwang 1985).

In general, the results suggest that the substitution in the aryl ring linked to the hydrazone moiety does not influence the activity of pyrazolylhydrazone compounds, since compound 11 (unsubstituted) showed similar activity to compounds 1, 4, 5, 7 and 12 (with different substituents).

Based on the structural characteristics of the pyrazolylhydrazone derivatives, we suggest that these compounds interfere in the arachidonic acid metabolism via cyclooxygenase, with subsequent reduction of thromboxane A_2 synthesis and inhibition of collagen-induced platelet aggregation.

Arachidonic acid induces platelet aggregation mainly by thromboxane A_2 synthesis (Vargaftig & Zirinis 1973).

Compounds 2, 1 and 11, at 100 μM, were the most active inhibitors of the arachidonic acid-induced platelet aggregation. These results indicate that the presence of an aryl ring linked to the hydrazone moiety with an adequate electronic density due to the nature of its substituents (i.e. furyl in compound 2), seems to be an important requirement for inhibitory activity. In contrast, the introduction of a vinylogue spacer unit between the hydrazone and the furyl moieties (compound 3) drastically reduces the inhibitory activity. These results suggest a weak interaction between compound 3 and its receptor site in view of the fact that the presence of a vinylogue spacer could be contributing to a steric separation between two important pharmacophoric sites in the active molecules.

The overall results obtained with compound 1 indicate that the addition of a nitro group at the *o*-position of a substituted ring confers a less specific inhibitory activity, since this compound demonstrated a marked activity on ADP-, collagen- and arachidonic acid-induced platelet aggregation. Compound 1 because of this may not be interacting with specific receptors on the platelet aggregation assay, even showing a potential anti-oedemic activity (Pereira 1989).

Compound 2 showed a high inhibitory activity against collagen- and arachidonic acid-induced platelet aggregation and it showed reduced activity in the ADP assays. Since this derivative did not show any anti-oedemic activity in preliminary studies with mice and rats (data not shown), we suggest that it probably has an inhibitory action on the arachidonic acid metabolism at a different site from that of cyclooxygenase.

The present findings, in association with those reported previously (Silveira et al 1991), showed that compound 2, 2-formylfurane-5-(1-phenyl-3-methyl-4-nitropyrazoylyl) hydrazone, was the most selective inhibitor of arachidonic acid-induced in-vitro platelet aggregation.

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