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THE INFLUENCE OF SOME 3-AMINO-2-PYRAZOLINE DERIVATIVES ON CYCLOOXYGENASE AND LIPOXIDASE ACTIVITIES

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Abstract—The influence of 3-amino-1-[*m*-(trifluoromethyl)-phenyl]-2-pyrazoline (BW 755C), 1-(3,3-diphenylpropyl)-3-amino-5-methyl-2-pyrazoline (KD 785) and 1-(3,3-diphenylpropyl)-3-amino-2-pyrazoline (KD 679) on cyclooxygenase and lipoxidase activities has been studied. All three compounds inhibited soybean and platelet lipoxidase activity. BW 755C was a much stronger inhibitor than KD compounds. The tested compounds stimulated oxygen consumption by cyclooxygenase from ram seminal vesicle microsomes in the presence of 100 μM of arachidonic acid in the range of concentrations between 30 and 300 μM and increased PGE_2 generation in these experimental conditions. Radiochemical studies showed that BW 755C had been a stimulator of cyclooxygenase in higher (100 μM) substrate concentrations but, in agreement with a previous report it acted as an inhibitor of this enzyme when substrate concentration was 1.6 μM. Probably this compound acted as a free radical scavenger.

Arachidonic acid is oxidized in animal tissues along two pathways: prostaglandin synthase (EC 1.14.99.1) and lipoxidases (EC 1.13.11.12).

The compound BW 755C (3-amino-1-[*m*-(trifluoromethyl)-phenyl]-2-pyrazoline) was claimed to be an inhibitor of both cyclooxygenase and lipoxidase from blood platelets [1]. It possesses also anti-inflammatory activity which comprises inhibition of leukocyte migration into inflamed areas [2]. The compounds which inhibit only cyclooxygenase enhance leukocyte migration [2].

Here we compare the influence of BW 755C and its two derivatives [3] (Fig. 1) on enzymic oxidation of arachidonic acid *in vitro*.

MATERIALS AND METHODS

3-Amino-1-[*m*-(trifluoromethyl)-phenyl]-2-pyrazoline (BW 755C) was synthesized at Wellcome Research Laboratories (Beckenham, U.K.)

1-(3,3-Diphenylpropyl)-3-amino-5-methyl-2-pyrazoline (KD 785) and 1-(3,3-diphenylpropyl)-3-amino-2-pyrazoline (KD 679) were synthesized at Chinoin Laboratories (Budapest, Hungary).

Arachidonic acid and soybean lipoxidase were bought from Sigma (St. Louis, MO), [1-^{14}C]arachidonic acid from the Radiochemical Centre (Amersham, U.K.) (batch 19; 61 mCi/mmole).

Prostaglandin E_2 was a gift from the Upjohn Chemical Company (Kalamazoo, MI).

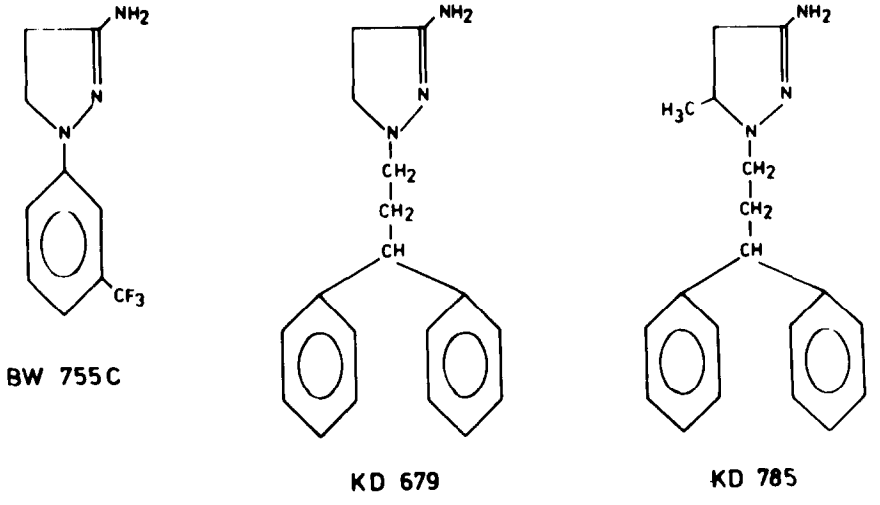

Fig. 1. Chemical structure of tested compounds.

Measurement of lipoxidase activities. Two lipoxidase preparations were used: crystalline soybean enzyme (15-lipoxidase, linoleate: oxygen oxidoreductase) and fresh 100,000 *g* supernatant from horse blood platelet homogenate which contained crude 12-lipoxidase. The activities of both preparations were measured as μmoles O_2 consumed in the presence of 100 μM of arachidonic acid as described previously [4]. Oxygen consumption in the presence of tested compounds was compared with that in control sample and expressed as per cent of inhibition. Regression lines were constructed and IC_{50} values calculated.

Measurement of cyclooxygenase activity (prostaglandin synthase). Lyophilized ram seminal vesicle microsomes (0.5 mg/ml) were suspended in 0.1 M phosphate buffer pH 7 and incubated with or without tested compounds in an oxymeter chamber as described previously [5]. Reaction velocity was calculated from the oxygen consumption during 3 min starting from the moment when arachidonic acid was added. The samples were then boiled. The amount of PGE_2 formed was bioassayed using rat stomach strip [6]. Oxygen consumption and the amount of PGE_2 formed in the presence of tested compounds were compared with those in their absence and expressed as per cent of control.

In other sets of experiments ram seminal vesicle microsomes were incubated exactly as for polarographic measurements but with addition to 1 ml sample: 1.64 nmoles (0.1 μCi) of [1-^{14}C]arachidonic acid with or without 100 nmoles of unlabelled arachidonic acid. Incubation mixture was acidified (pH 3) after 10 min incubation and extracted with ethyl acetate. The extract was put on TLC plates together with unlabelled standards of 6-oxo$PGF_{1\alpha}$, PGE_2, $PGF_{2\alpha}$ and PGD_2 and arachidonic acid. After development of the plates the standards were visualized in iodine vapour and compared with radioactive spots presented in autoradiochromatogram. The spots were scraped off and counted using liquid scintillation spectrometer Intertechnique SL-30 as previously described [7].

Cyclooxygenase activity in horse blood platelets was estimated radiochromatographically. Platelets were separated as described previously [4]. After freezing and thawing they were suspended in Tris–HCl buffer pH 7.5. Incubation mixture contained in 1 ml: platelet homogenate equivalent to 25 mg of protein (biuret method), 30 nmoles of BW 755C (or water in control) and 0.1 μCi of [^{14}C]arachidonic acid with or without 100 nmoles of unlabelled arachidonic acid. Extraction and TLC procedure were the same as in the case of ram seminal vesicle microsomes.

The influence of all three 3-aminopyrazoline derivatives on platelet aggregation was tested in platelet rich plasma of rabbit, containing 0.38% sodium citrate using Born's aggregometer [8]. Arachidonic acid (100 μM) was used as a pro-aggregatory agent.

RESULTS

Soybean lipoxidase activity was 9.8 ± 0.5 μmoles of O_2/min/mg of enzyme (mean ± SE), $n = 33$. 3-Aminopyrazoline derivatives inhibited this activity; BW 755C in the range of concentrations from 3 to 90 μM showed $IC_{50} = 11$ μM ($n = 20$, $r = 0.87$). BW 755C at a concentration of 1000 μM showed a lower degree of enzymic inhibition than at the concentration of 100 μM. KD 679 in the range of concentrations from 100 to 1000 μM showed IC_{50} value 670 μM ($n = 10$, $r = 0.90$) and KD 785 in the same range of concentrations—555 μM ($n = 8$, $r = 0.94$).

Activity of horse platelet lipoxidase was: 6.4 ± 0.4 nmoles of O_2/min/ml of the sample ($n = 25$).

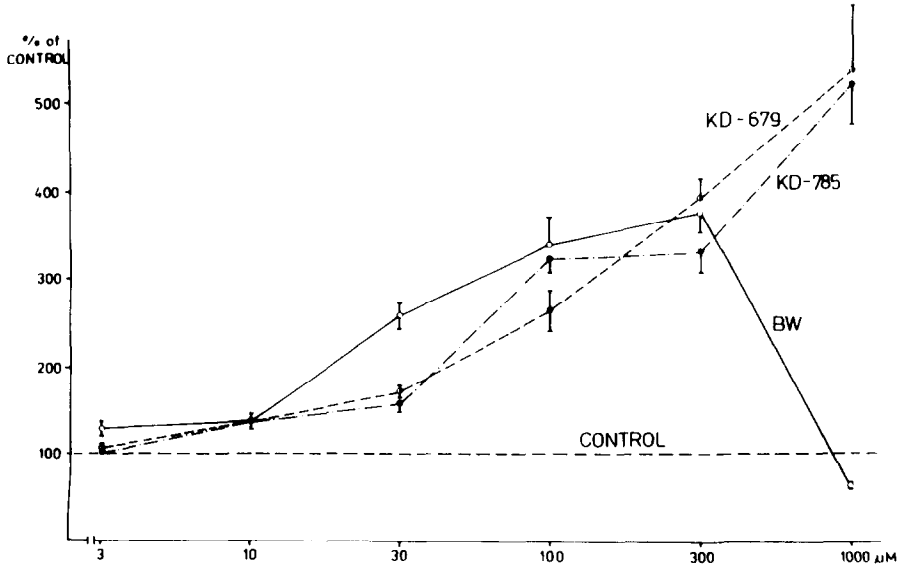

Fig. 2. The influence of tested compounds on oxygen consumption by ram seminal vesicle microsomes. Incubation mixture contains: 0.5 mg/ml of ram seminal vesicle microsomes suspended in 0.1 M phosphate buffer pH 7, 100 μM of arachidonic acid and various concentrations of tested compounds. Ordinate: per cent of control. Abscissa: log of the concentration of tested compounds. Each point represents the results of at least three experiments. S.E.M. is shown in the figure.

Table 1. The influence of tested compounds on prostaglandin generation in samples prepared as described in Fig. 2

Concentrations of tested compound	BW 755C	KD 679 (% of control)	KD 785
30 μM	255 ± 19.2 (4)	162 ± 18.0 (5)	247 ± 13.0 (3)
300 μM	146 ± 1.8 (4)	389 ± 45.9 (5)	383 ± 43.9 (3)

Postaglandin E_2 like activity was bioassayed.
Number of samples in parentheses.

BW 755C in the range of concentrations from 0.3 to 100 μM showed IC_{50} value 1.7 μM ($n = 9$, $r = 0.86$). KD compounds were much weaker. When tested in the range of concentrations between 100 and 5000 μM KD 679 and KD 785 have IC_{50} values 1170 μM ($n = 14$, $r = 0.82$) and 1510 μM ($n = 15$, $r = 0.82$) respectively.

Suspension of ram seminal vesicle microsomes consumed 9.0 ± 0.25 nmoles of O_2/min/mg of microsomes in the presence of 100 μM of arachidonic acid ($n = 48$). The influence of tested compounds on oxygen consumption by this suspension is shown in Fig. 2. They stimulated this consumption especially in the range of concentrations between 30 and 300 μM. BW 755C at a concentration of 1000 μM was an inhibitor of oxygen consumption whereas KD compounds showed the stronger stimulation at this concentration.

PGE_2 generation by ram seminal vesicle microsomes was 0.4 ± 0.02 nmoles of PGE_2/min/mg of microsomes ($n = 11$) in the absence of tested compounds. In their presence at the concentrations of 30 and 300 μM stimulation of PGE_2 production was observed (Table 1). BW 755C was a stronger stimulator at a concentration of 30 μM than at 300 μM. Conversely, KD compounds were stronger stimulators at a concentration of 300 μM. The stimulation of cyclooxygenase from ram seminal vesicle microsomes by tested compounds was confirmed radiochromatographically. The results show (Table 2) that all cyclooxygenase products were increased by only PGE_2 significantly. Conversion of arachidonic acid increased significantly on the influence of all three tested compounds (Table 2).

In the experiments in which only labelled arachidonic acid was used, BW 755C acted as an inhibitor of prostaglandin generation (Table 3). It significantly decreased $PGF_{2\alpha}$, PGE_2, and PGD_2 generation and increased the amount of unreacted arachidonic acid.

Comparing the amount of unreacted arachidonic acid in control samples with and without unlabelled arachidonic acid (Tables 2 and 3) it is evident that 100 μM is an excess of substrate since in its presence conversion of arachidonic acid is much lower. In the experiments with platelet homogenate (Table 4) conversion of arachidonic acid was high even in the

Table 2. Radiochemical evaluation of the influence of tested compounds on prostaglandin generation by ram seminal vesicle microsomes in the presence of 101.6 μM of arachidonic acid

	% Radioactivity determined as				
	6-oxo$PGF_{1\alpha}$	$PGF_{2\alpha}$	PGE_2	PGF_2	AA
Control	2.1 ± 0.2 (4)	1.9 ± 0.1 (4)	3.8 ± 0.4 (4)	3.7 ± 0.7 (4)	66.7 ± 3.0 (4)
BW 755C (30 μM)	2.2 ± 0.3 (3)	2.4 ± 0.4 (3)	10.6 ± 2.0* (3)	3.8 ± 0.5 (3)	56.3 ± 2.7* (3)
KD 679 (300 μM)	3.4 ± 0.3 (3)	3.4 ± 0.8 (3)	9.3 ± 2.0* (3)	5.6 ± 0.9 (3)	44.8 ± 5.3* (3)
KD 785 (300 μM)	3.2 ± 0.4 (3)	3.6 ± 0.8 (3)	10.9 ± 2.5* (3)	5.7 ± 1.2 (3)	49.0 ± 4.5* (3)

Incubation mixture contains the same composition as for polarographic measurements, with addition of 0.1 μCi [^{14}C]arachidonic acid. For experimental details see text.
Each result is the mean of at least three experiments (number of experiments in parentheses).
* Statistically significant: $0.05 > P > 0.01$.

Table 3. Radiochemical evaluation of the influence 30 μM BW 755C on prostaglandin generation by ram seminal vesicle microsomes in the presence of 1.6 μM of arachidonic acid

	% Radioactivity determined as				
	6-oxo$PGF_{1\alpha}$	$PGF_{2\alpha}$	PGE_2	PGD_2	AA
Control	3.7 ± 0.2	11.5 ± 2.8	20.9 ± 2.6	7.5 ± 1.5	22.0 ± 8.6
BW 755C	4.0 ± 0.9	7.0 ± 1.3	10.9 ± 2.6*	3.4 ± 0.2*	48.9 ± 5.6*

Experimental conditions were the same as presented in Table 2 except that unlabelled arachidonic acid was omitted.
Each result is the mean of three experiments.
* Statistically significant: $0.05 > P > 0.01$.

Table 4. Radiochemical evaluation of the influence of 30 μM BW 755C on prostaglandin generation by homogenate of horse platelet microsomes

% Radioactivity determined as:	1.6 μM of AA		101.6 μM of AA	
	Control	BW 755C	Control	BW 755C
6-oxo$PGF_{1\alpha}$	1.0 ± 0.1	0.9 ± 0.1	1.8 ± 0.2	1.6 ± 0.3
$PGF_{2\alpha}$	4.4 ± 1.5	1.9 ± 0.6	3.9 ± 1.8	4.0 ± 1.1
PGE_2	13.5 ± 2.8	4.7 ± 0.6*	13.9 ± 1.9	13.9 ± 1.0
PGD_2	2.1 ± 1.2	2.6 ± 1.0	6.4 ± 1.3	5.6 ± 0.7
AA	9.0 ± 3.9	18.8 ± 2.7	17.0 ± 3.9	13.0 ± 3.2

Incubation mixture contains horse platelet homogenate, water or BW 755C solution and 0.1 μCi [^{14}C]arachidonic acid with or without labelled arachidonic acid (in final concn 100 μM).

Each result is the mean of three experiments.

* Statistically significant: $0.05 > P > 0.01$.

presence of 100 μM of arachidonic acid. This concentration of substrate completely abolished inhibition of cyclooxygenase by BW 755C observed in the presence of low substrate concentration. In contrast to ram seminal vesicle microsomes, BW 755C was not a stimulator of cyclooxygenase in this enzymic preparation, probably because 100 μM of arachidonic acid was not the excess for this enzyme.

BW 755C and KD compounds had antiaggregatory activity. BW 755C was much stronger in this respect. It abolished completely arachidonic acid-induced platelet aggregation at a threshold concentration of 5.6 ± 1.6 μM ($n = 4$) whereas KD 679 and KD 785 needed 153 ± 13 μM ($n = 3$) and 67 ± 16.2 μM ($n = 5$) respectively.

DISCUSSION

It was confirmed that BW 755C is a potent inhibitor of lipoxidases. Its potency surpasses by far lipoxidase inhibition induced by two other *N*-substituted 3-amino-pyrazoline derivatives. A separation of 3-amino-pyrazoline ring from a phenyl moiety with a propyl chain in those derivatives also changes their selectivity towards two tested lipoxidases as compared with BW. BW 755C is a much stronger inhibitor of platelet lipoxidase than of soybean lipoxidase. Conversely, KD compounds are about two times weaker inhibitors of the first enzyme. Much greater potency of BW 755C as compared with KD compounds may also be the result of a trifluoromethyl substituent in BW 755C.

The stimulation of cyclooxygenase activity in RSVM by all three 3-amino-pyrazoline derivatives was rather unexpected. BW 755C was described [1] as a stong inhibitor of cyclooxygenase in blood platelets. It also diminished prostaglandin generation in the rat sponge exudates [2]. Stimulation of cyclooxygenase was observed only when 100 μM of arachidonic acid was used. In the presence of 1.6 μM of arachidonic acid (labelled only) BW 755C acted, in agreement with Higgs's [1] experiments, as an inhibitor of cyclooxygenase.

Compounds which are free radical scavengers prevent inhibition of the enzyme by the excess of arachidonic acid [9] and it is why they activate cyclooxygenase [7, 9]. On the basis of the experiments presented in this paper we postulate that BW 755C acts as a free radical scavenger. It activates cyclooxygenase when free radicals are formed in excess (high ratio of the substrate to enzyme) but inhibits by the scavenging of small amounts of these radicals which are necessary for initiation of cyclooxygenation [10] (low ratio of the substrate to enzyme). This explanation is confirmed additionally by the fact that chlorpromazine, a known free radical scavenger [11] stimulates cyclooxygenase only in the presence of the excess of substrate [7] but inhibits when arachidonic acid concentration is 1.6 μM (our unpublished results).

Inhibition of lipoxidases by BW 755C is not correlated with free radical scavenging properties since some free radical scavengers do not influence lipoxidases [12] and some, e.g. chlorpromazine, inhibit them [4].

Higgs found that BW 755C lowered a prostaglandin level *in vivo* [2]. On the basis of these experiments it seems that the intracellular level of arachidonic acid is within the range in which BW 755C inhibits prostaglandin generation. This fact may explain the antiaggregatory properties of all tested compounds. Arachidonic acid was used in aggregation experiments in the concentration (100 μM) at which BW 755C should have stimulated generation of prostaglandins. This concentration was, however, only extracellular. On the basis of the antiaggregatory properties of BW 755C it is postulated that inside the platelets it is the substrate concentration in which BW 755C acts as an inhibitor. We found also (unpublished biological results) that no thromboxane A_2 was formed in platelet rich plasma in the presence of antiaggregatory concentration of BW 755C.

We conclude that inhibition of stimulation of cyclooxygenase by tested 3-amino-2-pyrazoline derivatives depends on a substrate concentration. Probably these compounds act as free radical scavengers.

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