Br. J. Pharmacol. (1988), **94**, 528–539

Selective inhibition of arachidonate 5-lipoxygenase by novel acetohydroxamic acids: biochemical assessment *in vitro* and *ex vivo*

J.E. Tateson, R.W. Randall, C.H. Reynolds, †W.P. Jackson, *P. Bhattacherjee, *J.A. Salmon & [1]L.G. Garland

Departments of †Medicinal Chemistry, Biochemical Sciences and *Pharmacology, Wellcome Research Laboratories, Langley Court, Beckenham, Kent, BR3 3BS

1 The chemically novel acetohydroxamic acids, BW A4C, BW A137C and BW A797C, are potent inhibitors of the synthesis of leukotriene B_4 (LTB_4) from arachidonic acid by human leucocyte homogenates: the concentrations required for 50% inhibition (IC_{50}) were 0.1 μM, 0.8 μM and 0.5 μM respectively. Inhibition was less at higher concentrations of arachidonic acid.

2 These compounds also inhibited the synthesis of [^{14}C]-5-HETE from [^{14}C]-arachidonic acid and the calcium-dependent synthesis of LTB_4 from 5-HPETE. This, therefore, suggests that they inhibit 5-lipoxygenase and LTA_4 synthase.

3 Concentrations of acetohydroxamic acids required to inhibit metabolism of arachidonic acid by cyclo-oxygenase, 12-lipoxygenase and 15-lipoxygenase were 10 to 100 times higher than those required to inhibit 5-lipoxygenase.

4 The compounds were potent inhibitors of LTB_4 synthesis induced by the ionophore, A23187, in human intact leucocytes. This inhibition was reversed by washing the cells. They were also potent, selective inhibitors of LTB_4 synthesis induced by A23187 in whole rat blood: binding to rat plasma proteins did not greatly reduce the effectiveness of the compounds.

5 The effects of the acetohydroxamic acids, administered either intravenously or orally to rats, on the synthesis of LTB_4, and thromboxane B_2 (TXB_2) in A23187-stimulated blood *ex vivo* was studied. The three compounds caused dose-dependent inhibition of the synthesis of LTB_4 but not TXB_2. Inhibition of LTB_4 synthesis persisted for up to 6 h after a single oral dose of 50 mg kg^{-1}.

6 The plasma concentrations of unchanged compound determined by h.p.l.c. correlated with the inhibition of LTB_4 synthesis *ex vivo*.

Introduction

A large body of experimental evidence suggests that products of the 5-lipoxygenase (5-LO) pathway of arachidonic acid metabolism may mediate some of the pathological events associated with acute inflammation and the reversible airways obstruction of asthma (Samuelsson, 1983; Higgs *et al.*, 1984; Lewis, 1985; Bray, 1986). For many years, therefore, there have been considerable efforts directed towards the discovery of inhibitors of this pathway, and a number of such inhibitors have already been described. Some compounds such as BW 755C inhibit cyclo-oxygenase (CO) and 5-LO with similar efficacy, while others such as nafazatrom and nordihydroguaiaretic acid (NDGA) inhibit the 5-LO reaction preferentially. Recently Corey *et al.* (1984) reported the competitive inhibition of 5-LO from RBL-1 cells by hydroxamic acid analogues of arachidonic acid. The present paper describes the inhibitory activities of BW A4C, BW A137C and BW A797C, three examples taken from a broad class of novel acetohydroxamic acid derivatives that preferentially block 5-LO rather than CO.

Several compounds are already known which block preferentially 5-LO *in vitro* e.g. eicosatetraynoic acid (ETYA; Hamberg & Samuelsson, 1974), NDGA (Hamberg, 1976) and nafazatrom (Mardin & Busse, 1983). Investigators have tested some of these compounds *in vivo* and have drawn conclusions as to

[1] Author for correspondence.

the involvement of 5-LO products in animal models of human disease (see Salmon, 1986). However, most of the compounds which inhibit 5-LO *in vitro* are either not bioavailable or are short-lived *in vivo*. It is clearly essential to confirm that compounds are bioavailable and have suitable biological half-lives before they are evaluated in animal models of human disease or in the clinic. Therefore, we have also evaluated the degree and duration of the inhibition of 5-LO after either oral or intravenous administration of acetohydroxamic acids to rats by monitoring the synthesis of leukotriene B_4 (LTB_4) in blood stimulated *ex vivo* with the calcium ionophore, A23187, using a procedure similar to that reported by McMillan *et al.* (1986) and Gresele *et al.* (1986). The selectivity of the compounds was assessed by measuring the CO product thromboxane B_2 (TXB_2) formed in the stimulated blood: the LTB_4 and TXB_2 were presumably biosynthesized by leucocytes and platelets, respectively. We have also determined whether binding of the acetohydroxamic acids to plasma protein could compromise their activities *in vivo*. In addition, the concentrations of unchanged acetohydroxamic acids in plasma after dosing were determined by high performance liquid chromatography (h.p.l.c.) with ultra-violet absorbance detection to examine whether these concentrations correlated with the inhibitory activities.

Methods

Human leucocyte homogenates

Fresh blood, from donors who had not ingested aspirin or other drugs for at least 14 days previously, was collected, with EDTA (1.2 mg ml^{-1}) as anticoagulant; erythrocytes were sedimented by addition of 2% methyl cellulose (7.5 ml 100 ml^{-1} blood) and incubation for 40 min at 37°C. The supernatant containing leucocytes and platelets was removed, diluted two fold with sodium phosphate-buffered saline (0.9% NaCl, pH 7.2, 20 mM phosphate) and centrifuged for 10 min at 150 *g*. The supernatant, containing platelets, was removed and the leucocyte pellet resuspended in 50 ml of ice-cold 0.82% NH_4Cl containing 5 mM KCl (brought to pH 7.4 with 4.4% $NaHCO_3$); this procedure lysed any remaining erythrocytes during 10 min incubation on ice. The leucocyte suspension was then centrifuged again (10 min, 150 *g*) and the pellet was resuspended in 25 ml sodium phosphate buffer (50 mM, pH 7.0) containing EDTA (1 mM). The cell density was determined, with a Coulter counter; differential counts made on some samples showed that the preparation contained mainly leucocytes, of which >80% were polymorphonuclear. Platelet counts were not made and no attempt was made to decrease further the platelet content of this cell suspension. The leucocyte-rich cell suspension was then centrifuged once more (10 min, 150 *g*) and the pellet was resuspended in sodium phosphate buffer (50 mM, pH 7.0) containing EDTA (1 mM) at a density of 10–20 × 10^6 cells ml^{-1}. Cells were homogenized by sonication (MSE Soniprep 150; 3 × 30 s, 22 micron amplitude setting) at 0°C. Normally reactions were carried out with the complete homogenate but, when appropriate, the cytoplasmic fraction was separated by centrifugation at 100,000 *g* for 60 min. Portions (0.47 ml) of homogenates were then warmed to 37°C and 10 μl of inhibitor or appropriate vehicle (usually dimethysulphoxide, DMSO) was added. After further incubation (usually 5 min) at 37°C the reactions were started by addition of arachidonic acid and $CaCl_2$ (10 μl of each; final concentrations 5 μM and 2 mM, respectively). Reactions were terminated after 5 min at 37°C by boiling (5 min). Reaction products, TXB_2 and LTB_4, were measured by specific radioimmunoassays (RIA) (Salmon, 1978; Salmon *et al.*, 1982). Blanks (boiled homogenate) and vehicle controls were included in each experiment and each reaction was carried out in triplicate. The concentration of arachidonic acid was chosen to yield approximately 60% of the maximum amount of LTB_4 produced by this system. The control levels of LTB_4 and TXB_2 (means ± s.e. mean from 20 experiments) were 27.0 ± 2.2 ng ml^{-1} and 21.0 ± 4.3 ng ml^{-1}, respectively.

Conversion of [^{14}C]-arachidonic acid (5 μM) to ^{14}C-labelled products was measured in incubations that were identical to the above except for the use of labelled substrate. Reactions were terminated by addition of 2.5 vol acid/acetone (acetone containing 1% (v/v) 5 M HCl). Lipids were extracted in $CHCl_3$, dried under N_2, redissolved in $CHCl_3$/MeOH (2 : 1 v/v) and separated by thin layer chromatography using diethyl ether/hexane/glacial acetic acid (60 : 40 : 1 v/v). ^{14}C-labelled products were located by autoradiography, removed by scraping and counted (Packard TriCarb). Enzyme activity was measured as percent conversion of [^{14}C]-arachidonic acid to [^{14}C]-5-hydroxy-6,8,11, 14-eicosatetraenoic acid ([^{14}C]-5-HETE).

In the t.l.c. system used, the R_F values of arachidonic acid and authentic metabolites were as follows: arachidonic acid (0.70); 12-HETE (0.61); 15-HETE (0.61); 5-HETE (0.40); LTB_4 (0.11).

Intact human leucocytes

Leucocytes were isolated essentially as described above but were, in addition, sedimented through Ficoll-Paque gradients to increase the purity of the

leucocytes (Palmer *et al.*, 1980). They were then suspended in indicator-free Hanks' balanced salts solution buffered to pH 7.4 with HEPES (30 mM). The cell suspension (5×10^6 cells ml^{-1}) was incubated at 37°C with inhibitor or vehicle (DMSO) before initiating the reaction with the calcium ionophore A23187 (1 $\mu g\,ml^{-1}$). Incubations were terminated after 5 min by centrifugation and supernatants decanted for measurement of reaction products. The control level of LTB_4 formed was 41.3 ± 5.6 ng ml^{-1} ($n = 12$). In contrast with the leucocyte-rich homogenates, the amount of TXB_2 formed by purified leucocytes was often low (<0.5 ng ml^{-1}) and inhibition of TXB_2 synthesis could not be assessed.

Human platelet homogenates

Platelets were separated from platelet-rich plasma by centrifugation (2,500 *g*; 15 min; 4°C) and resuspended in a volume of buffer (pH 7.0; sodium phosphate 50 mM, EDTA 1 mM, indomethacin 20 μM) equivalent to 4% of the original plasma volume. Platelets were homogenised by sonication (MSE Soniprep 150; 3 × 30 s, 22 micron amplitude setting) at 0°C. Measurements of 12-lipoxygenase (12-LO) activity were carried out in 0.43 ml sodium phosphate buffer (50 mM, pH 7.0) containing EDTA (0.4 mM) to which was added 10 μl inhibitor or DMSO (vehicle control) and 50 μl platelet sonicate. After 5 min preincubation the reaction was started by addition of 1 μg [^{14}C]-arachidonic acid in 10 μl ethanol; the final concentration of arachidonic acid was 6.6 μM. The reaction was allowed to proceed for 2 min at 37°C before being stopped by the addition of acid/acetone and lipids were extracted as above. Enzyme activity was measured as percentage conversion of [^{14}C]-arachidonic acid to [^{14}C]-12-hydroperoxy-5,8,10,14-eicosatetraenoic acid ([^{14}C]-12-HPETE) and [^{14}C]-12-hydroxy-5,8,10,14-eicosatetraenoic acid ([^{14}C]-12-HETE).

Stimulation of 5-lipoxygenase in rat whole blood

The method of stimulating 5-LO in rat whole blood used in our laboratories is similar to that described by McMillan *et al.* (1986) and Gresele *et al.* (1986). Blood was collected from male Wistar rats into plastic tubes containing heparin (10 u ml^{-1}). Within 2 min of collection, blood (0.5 ml) was added to 1.5 ml Eppendorf tubes which were placed in a water bath at 37°C. Calcium ionophore, A23187, was added in DMSO (10 μl per ml of blood) and incubation continued at 37°C. The results of preliminary experiments (not shown) established that the optimum concentration of A23187 was 15 $\mu g\,ml^{-1}$ and the optimum incubation time was 30 min; these conditions were used in all the experiments described in this paper. At the end of the incubation, samples were centrifuged (12,000 *g* for 2 min) and the cell-free plasma was removed and stored at −20°C until reaction products were analysed. The concentrations of LTB_4 and TXB_2 in the plasma were determined by specific RIA after suitable dilution of the sample (1 : 20–1 : 200) but without prior extraction or chromatography. In 30 experiments (at least 5 animals per experiment) the concentrations of LTB_4 and TXB_2 formed by rat whole blood stimulated by A23187 were 26.6 ± 1.5 ng ml^{-1} and 376 ± 22 ng ml^{-1} respectively; the coefficients of variation were 26.5% and 33.6%, respectively.

The identity of immunoreactive LTB_4 in a pooled sample of plasma from A23187-stimulated blood was confirmed to be mainly LTB_4 by subjecting an extract to h.p.l.c. prior to RIA, as described previously (Salmon *et al.*, 1982; McMillan *et al.*, 1986; Gresele *et al.*, 1986); in addition, a small amount of immunoreactivity eluted at the retention times of the 6-*trans*-diastereomers of LTB_4 and of 12-HETE. Also, BW 755C, which inhibits CO and 5-LO was added (final concentration 100 $\mu g\,ml^{-1}$) to an aliquot of control blood 10 min before addition of A23187 in each experiment in order to provide limited confirmation of the identity of LTB_4 and TXB_2 (i.e. it was confirmed that the formation of immunoreactive (i)-LTB_4 and i-TXB_2 in A23187-stimulated blood was inhibited by BW 755C). Appropriate vehicle controls were included in all experiments.

Inhibition of 5-lipoxygenase in rat whole blood in vitro *by acetohydroxamic acids*

The inhibitory activities of the acetohydroxamic acids against 5-LO in rat whole blood were compared with their activities in washed leucocytes in order to assess whether binding to plasma proteins reduced their potency. Untreated male Wistar rats (150–220 g) were narcosed by CO_2 and blood withdrawn; the heparinised blood from approximately 5 animals was combined. Aliquots (0.44 ml) of the pooled blood were transferred to individual Eppendorf tubes. Various concentrations of the test compound were added in 2.5% DMSO in Hanks' buffered salt solution (pH 7.4; 50 μl) to triplicate aliquots of blood. After vortex-mixing the samples were left at 37°C for 10 min, then incubated with A23187 as described below. The concentrations of LTB_4 and TXB_2 in the cell-free plasma were determined by specific RIA (see above).

Inhibition of 5-lipoxygenase in rat whole blood ex vivo *by acetohydroxamic acids*

Wistar rats (150–200 g; 6–8 weeks old) were deprived of food for 12 h before administration of drugs. Drug

solutions or relevant controls were administered in a volume of 2.5 ml kg^{-1} orally or 1 ml kg^{-1} into the tail vein; the vehicle for oral administration was polyethylene glycol 300 and that for i.v. injection was 50% DMSO in 0.9% saline. Groups of animals were narcosed with CO_2 and blood collected at intervals (2 min–10 h) after administration of drug or vehicle. There were at least 5 animals in each group.

Within 2 min of collection, duplicate samples (0.5 ml) of blood were stimulated with A23187 (15 $\mu g\,ml^{-1}$) for 30 min at 37°C. The amounts of LTB_4 and TXB_2 formed were determined by RIA. The biosynthesis of LTB_4 and TXB_2 in blood from control groups of rats (given vehicle only) was evaluated at two time points (0 and 6 h) in each experiment. The concentrations of the eicosanoids synthesised in blood after administration of test compound were expressed as a percentage of the mean control level in the relevant experiment. Significance of difference from the control concentration was assessed by Student's *t* test.

In addition to the experiments described above, the degree of inhibition of LTB_4 synthesis achieved after oral dosing with the acetohydroxamic acids was compared with that obtained with a related hydroxamic acid, BW A509C.

Determination of acetohydroxamic acids in plasma

(*a*) *Extraction* Internal standard (see below) and ice-cold acetone (1 ml) were added to the sample of plasma (0.5 ml) which was vortex-mixed for 20 s and then centrifuged at 1500 *g* for 5 min. The supernatant was decanted into a second test-tube containing 0.2 ml saturated sodium chloride solution and extracted with chloroform (1.5 ml). The chloroform was removed under a stream of nitrogen and the residue was redissolved in tetrahydrofuran (THF): water (40 : 60 v/v; 250 μl). *N*-(5,6,7,8-tetrahydro-2-naphthylallyl)pivalohydroxamic acid, used as internal standard in the assay of both BW A797C and BW A4C, was added to the plasma samples at a concentration of 2 $\mu g\,ml^{-1}$. *N*-[2-(5,6,7,8-tetrahydro-2-naphthyloxy)ethyl] pivalohydroxamic acid, at a concentration of 5 $\mu g\,ml^{-1}$ plasma, was used as internal standard in the assay of BW A137C.

Calibration curves were obtained by adding the test compound and appropriate internal standard to plasma obtained from untreated rats, and these control samples were processed as for test samples.

(*b*) *High performance liquid chromatography* Aliquots (50 μl) of the extracts in THF : water (40 : 60 v/v) were injected with a Wisp 712 autoinjector (Waters Associates, Harrow, Middlesex) on to a Spherisorb octadecasilyl (ODS) 5 μm column (Laboratory Data Control, Stone, Staffs) which was maintained at 30°C with a column block heater (Jones Chromatography Ltd., Llanbradach, Glamorgan). Mobile phase was pumped through the column at 1 ml min^{-1} by use of a Waters Associates Model 6000 A delivery system. Eluted compounds were detected with a Spectromonitor III variable wavelength u.v. absorbance detector (Laboratory Data Control). For the assays of both BW A4C and BW A797C the mobile phase was THF : water : trifluoroacetic acid (TFA) (50 : 50 : 0.1; v/v/v) containing 0.5 mM oxalic acid and the eluate was monitored at 260 nm. The mobile phase used for the assay of BW A137C was THF : water : TFA (47.5 : 52.5 : 0.1, v/v/v) containing 0.5 mM oxalic acid and the eluate was monitored at 226 nm.

Peak areas were integrated with a Hewlett-Packard 3357 computer system which was also used to calculate the concentration of the compounds using the calibration curves (the ratio of integrated peak areas was plotted against concentration).

Materials

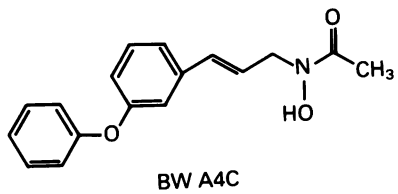
Arachidonic acid (free acid, 99% pure), ionophore A23187 (free acid) and NDGA were obtained from Sigma UK; t.l.c. plates LK5D were obtained from Whatman; autoradiography films DEF2 from Kodak; Ficoll-Paque from Pharmacia and methyl cellulose, low substitution, from BDH (Poole, Dorset); [1-^{14}C]-arachidonic acid (55 mCi $mmol^{-1}$), [^{3}H]-leukotriene B_4 (170 Ci $mmol^{-1}$) and [^{3}H]-thromboxane B_2 (170 Ci $mmol^{-1}$) were from Amersham International; 12(S,R)-HETE, 5(S,R)-HETE and 5(S,R)-HPETE were synthesized at the Wellcome Research Laboratories as previously described (Palmer *et al.*, 1980). Nafazatrom and BW755C were also synthesized at the Wellcome Research Laboratories. Analytical and h.p.l.c. grade solvents were from BDH and Rathburn Chemicals Limited (Walkerburn, Peebleshire) respectively.

The three acetohydroxamic acid derivatives BW A4C, BW A137C and BW A797C were synthesized as described by Jackson *et al.* (1988). The structures of these compounds are as follows: BW A4C, N-(3-phenoxycinnamyl)-acetohydroxamic acid; BW A137C, N-(4-benzyloxybenzyl)-acetohydroxamic acid; BW A797C, N-[3-(5,6,7,8-tetrahydro-2-naphthyl)prop-2-enyl]-acetohydroxamic acid (see Figure 1). A related hydroxamic acid, BW A509C (3-phenoxy-N-methylcinnamohydroxamic acid), was also synthesized by Jackson *et al.* (1988).

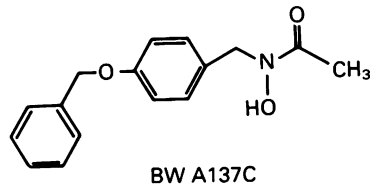
Results

Leucocyte-rich homogenates

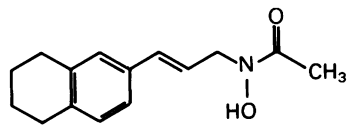
The three acetohydroxamic acids (BW A4C, BW



BW A4C



BW A137C



BW A797C

Figure 1 Structures of BW A4C, BW A137C and BW A797C.

A137C, BW A797C) inhibited the synthesis of LTB_4 at lower concentrations than were required to inhibit synthesis of TXB_2 (Figure 2); in this respect they resemble nafazatrom and NDGA. Low concentrations of each selective inhibitor increased the production of TXB_2 while inhibiting its synthesis at higher concentrations. The maximum increase in TXB_2 production caused by the acetohydroxamic acids was not significantly above control, whereas the maximum increase in TXB_2 due to NDGA and nafazatrom was significant ($P < 0.05$, 2-tailed t test). This effect of the selective inhibitors was not investigated further. As described previously (Randall *et al.*, 1980), BW 755C inhibited equally the synthesis of TXB_2 and LTB_4 and showed no increase in TXB_2 synthesis.

Each acetohydroxamic acid also blocked the metabolism of [^{14}C]-arachidonic acid to [^{14}C]-5-HETE and the concentrations required were similar to those required to inhibit LTB_4 production in the same experiment (Table 1). Furthermore, BW A137C also inhibited the calcium-dependent synthesis of immunoreactive LTB_4 from 5(S,R)-HPETE by the supernatant from leucocyte homogenates (Figure 3). Similar results were obtained with BW A4C (not shown). The inhibitory effect of the acetohydroxamic acids was decreased by increasing the concentration of arachidonic acid (not shown). This applied also for the standard compounds tested in the same experiment, namely BW 755C, NDGA and nafazatrom.

Intact human leucocytes

Each of the three novel acetohydroxamic acids inhibited A23187-induced synthesis of LTB_4 in human leucocytes; effective concentrations were similar to those in leucocyte-rich homogenates (Table 1). Inhibition of LTB_4 synthesis was reversed

Table 1 Effect of acetohydroxamic acids and other inhibitors on the synthesis of 5-lipoxygenase and cyclooxygenase products by either human leucocytes or rat whole blood *in vitro*

	Human				*Rat*	
	a *Leucocyte-rich homogenates* (n = 3–9)			**b** *Intact leucocytes* (n = 2)	**c** *Whole blood* (n = 1)	
	[^{14}C]-*5-HETE*	LTB_4	TXB_2	LTB_4	LTB_4	TXB_2
BW A4C	0.3 ± 0.2	0.1 ± 0.03	3.2 ± 0.8	0.04	0.1	16.6
BA A137C	1.3 ± 0.8	0.8 ± 0.2	22.0 ± 4.0	0.4	0.1	32.4
BW A797C	0.5 ± 0.2	0.5 ± 0.1	5.8 ± 1.1	0.2	0.4	45.9
BW 755C	—	19.0 ± 4.0	14.0 ± 4.0	5.4	31.0	4.0
NDGA	—	0.8 ± 0.2	10.0	0.5	2.0	>27
Nafazatrom	—	6.4 ± 1.3	100.0	1.6	23.1	>81

Synthesis was stimulated by adding (a) arachdonic acid and $CaCl_2$ or (b and c) the ionophore A23187. In (b) the synthesis of TXB_2 was not detectable. The results shown are IC_{50} values derived from concentration-effect curves: where indicated values are mean ± s.e. mean from (n) separate experiments.

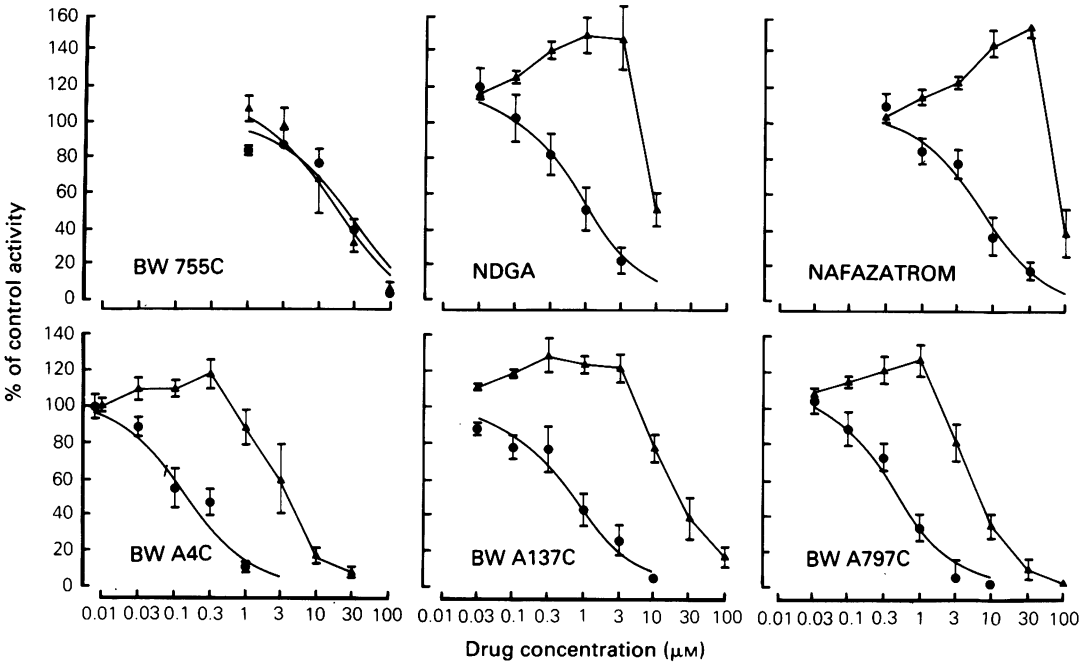


Figure 2 Effect of various compounds on the synthesis of leukotriene B_4 (LTB_4) and thromboxane B_2 (TXB_2) in homogenates of human leucocytes (equivalent to 10^7 cells per 0.5 ml) incubated with inhibitors (5 min at 37°C) before the simultaneous addition of arachidonic acid and $CaCl_2$ (final concentrations 5 μM and 2 mM, respectively). Reactions (5 min, 37°C) were terminated by boiling and products measured by RIA (LTB_4 ●, TXB_2 ▲). Each point is the mean of 3–5 experiments with vertical lines showing s.e. mean.

by washing the cell suspension with fresh medium before addition of ionophore (Figure 4). Since the amount of TXB_2 formed in these incubations with highly purified leucocytes was often low, the inhibition of TXB_2 synthesis could not be assessed.

Selectivity of inhibitors

The selectivity of the novel lipoxygenase inhibitors was evaluated further by monitoring their effects on the conversion of [^{14}C]-arachidonic acid to products of the 15-lipoxygenase (15-LO) and 12-LO pathways of metabolism in leucocytes stimulated with A23187 and of 12-LO in homogenates of human platelets. In A23187-stimulated leucocytes the acetohydroxamic acids were more effective inhibitors of the synthesis of 5-HETE and di-HETEs, including LTB_4, than of the synthesis of 12-HETE and 15-HETE (the radioactive zone with an R_F value of 0.61 was confirmed by h.p.l.c. analysis to be a mixture of 12-HETE and 15-HETE, as found previously by Borgeat & Samuelsson (1979)). The IC_{50} values for inhibition of the synthesis of 12-HETE/15-HETE were for BW A4C, 3.3 μM, for BW A137C, 15 μM, and for BW A797C, 6 μM. These values are about 20 times higher than IC_{50} values for inhibition of the synthesis of 5-LO products. The synthesis of [^{14}C]-12-HETE from [^{14}C]-arachidonic acid added to human platelet homogenates was also inhibited by BW A4C and BW A137C, the IC_{50} values being 1.2 ± 0.3 μM ($n = 3$) and 2.2 ± 0.4 μM ($n = 4$) respectively (these values being mean ± s.e. mean from n experiments).

Effect of acetohydroxamic acids on 5-lipoxygenase in rat blood in vitro

The effects of acetohydroxamic acids and other compounds on 5-LO and CO activity in blood *in vitro* are summarised in Table 1. The preferential inhibition of 5-LO exhibited by BW A4C, BW A137C and BW A797C in human leucocytes was also observed in rat whole blood; the ratios of the IC_{50} values against 5-LO and CO in whole blood were more than 100. The inhibitory potencies of the acetohydroxamic acids against 5-LO in rat blood were only 2–5 times higher than those in human isolated leucocytes, suggesting that binding to plasma protein does not significantly reduce the effectiveness of the compounds in rat blood. Similar results were obtained with human whole blood (data not shown).

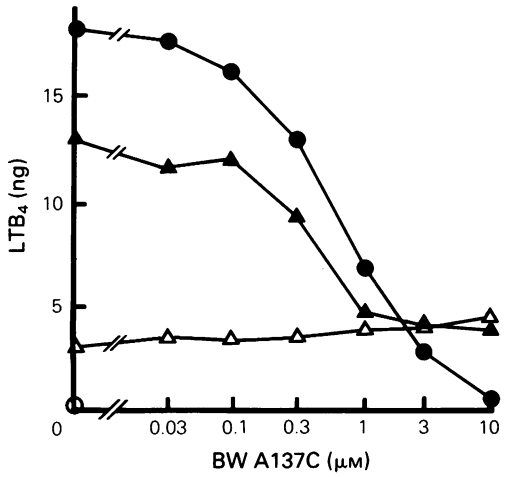


Figure 3 Effect of BW A137C on immunoreactive leukotriene B_4 (LTB_4) production from arachidonic acid and 5-HPETE. High speed (100,000 *g*) supernatant from human leucocytes (equivalent to 5×10^6 cells per 0.5 ml aliquot) was incubated in 50 mM sodium phosphate (pH 7) with 0.4 mM EDTA plus 0.6 mM $CaCl_2$ (●,▲) or 1 mM EDTA (○,△) at 30°C. Reactions were initiated with 6.6 μM arachidonic acid (●,○) or 3.6 μM 5(S,R)-HPETE (▲,△) and terminated after 2 min by boiling. LTB_4 was measured by radioimmunoassay.

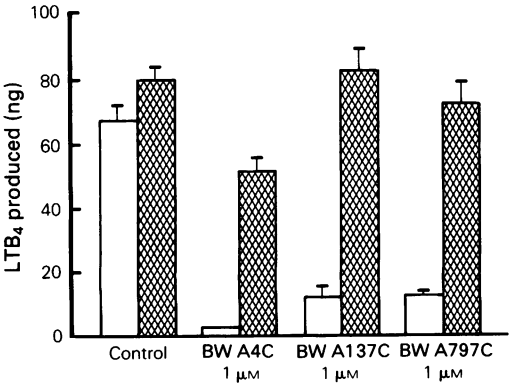


Figure 4 Reversal of 5-lipoxygenase (5-LO) inhibition by washing. Aliquots of a leucocyte-rich suspension (10^7 cells ml^{-1} in HEPES-buffered Hanks' solution, $CaCl_2$ 1 mM) were incubated (5 min, 37°C) with various inhibitors before half of each sample was washed once by centrifugation and resuspension. Washed (cross-hatched columns) and unwashed (open columns) cells were then stimulated with ionophore A23187 (1 μM) and the reaction (5 min, 37°C) terminated by centrifuging; leukotriene B_4 (LTB_4) contents of supernatants were measured by RIA. Each column represents mean of 3 experiments with s.e. mean shown by vertical lines.

Effect of acetohydroxamic acids and other compounds on 5-lipoxygenase activity in blood ex vivo

The onset and persistence of inhibition of LTB_4 and TXB_2 synthesis in blood stimulated with A23187 *ex vivo* after oral administration of three standard compounds is shown in Figure 5. The synthesis of the CO product TXB_2 was abolished soon after the administration of flurbiprofen (50 mg kg^{-1}, p.o.) and this effect lasted for at least 6 h; however, this high dose of flurbiprofen produced no significant reduction of the synthesis of the 5-LO product, LTB_4. BW 755C (50 mg kg^{-1}, p.o.) reduced the synthesis of both TXB_2 and LTB_4. NDGA (50 mg kg^{-1}) did not significantly affect formation of either LTB_4 or TXB_2 in blood at any time (0–6 h) after oral administration (not shown). Nafazatrom (50 mg kg^{-1}) caused a modest inhibition of LTB_4-synthesis but only during the first hour after oral dosing.

The three acetohydroxamic acids inhibited synthesis of LTB_4, but not TXB_2, after oral administration (Figure 5). The failure to inhibit synthesis of the CO product TXB_2 confirmed the selective action of these compounds. At a dose of 50 mg kg^{-1} p.o., the compounds BW A4C and BW A797C were effective for more than 6 h; compound BW A137C was less persistent. The duration of action of these three compounds was explored further by examining ED_{50} values for inhibition of LTB_4 synthesis at various time intervals after oral dosing (Table 2). The ED_{50} values, derived from log dose-response curves, confirmed the more persistent activity of BW A4C and BW A797C compared with BW A137C.

The three novel acetohydroxamic acids also inhibited LTB_4-synthesis in blood *ex vivo* after a single bolus intravenous injection (2 mg kg^{-1}; data not shown); the inhibition persisted for 2 h (BW A137C, BW A797C) or longer than 4 h (BW A4C).

The acetohydroxamic acids were more potent and persistent than a corresponding hydroxamic acid, BW A509C (data not shown); Figure 6 illustrates the dose-response curves for the inhibition of LTB_4-synthesis *ex vivo* by BW A4C and BW A509C administered p.o. 3 h before assessment.

Measurements of acetohydroxamic acids in plasma

The analytical methods described permit accurate and reproducible determination of the acetohydroxamic acids in plasma. The calibration graphs were linear over the range 0–50 μg ml^{-1}. The presence of minor interfering peaks limited the sensitivity of the assays of BW A4C and BW A797C to 0.1 μg ml^{-1} plasma and that of BW A137C to 0.2 μg ml^{-1}. The efficiency of the extraction procedure was greater than 80% (determined by comparing peak areas

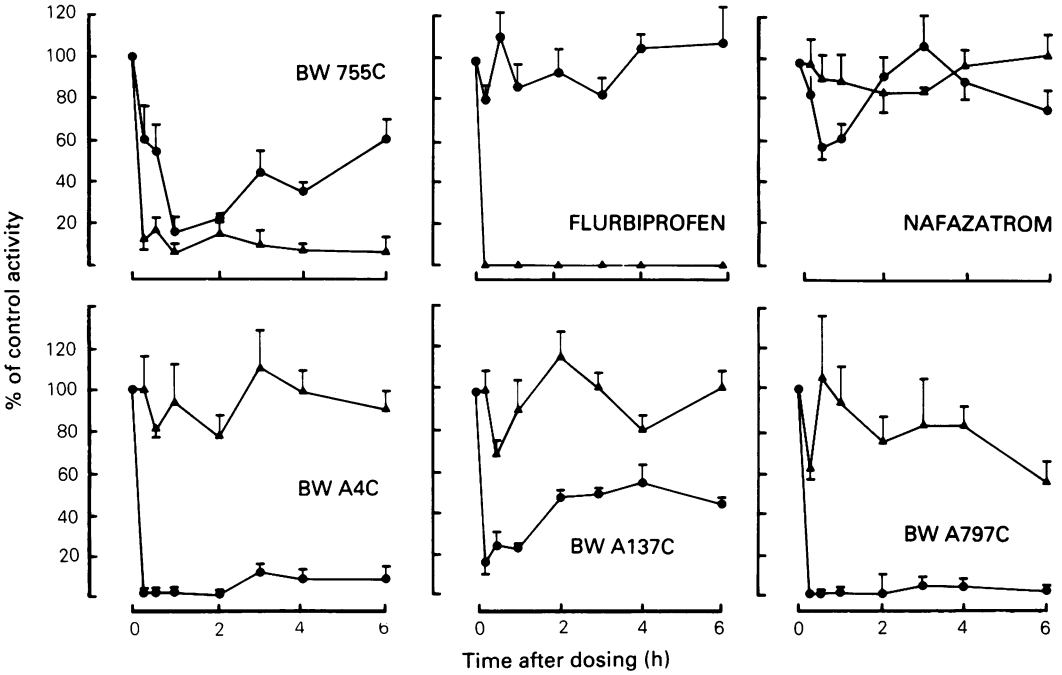


Figure 5 Effect of various compounds on the *ex vivo* synthesis of leukotriene B_4 (LTB_4) and thromboxane B_2 (TXB_2) in rat whole blood stimulated with A23187. Each compound was administered at a dose of 50 mg kg^{-1}, p.o. (●) LTB_4, (▲) TXB_2. The mean of data from 5 rats is plotted; vertical lines show s.e. mean.

from non-extracted and extracted standards). The inter-assay coefficients of variation for the assay of BW A4C in plasma were 10.3% and 8.5% at the concentrations of 0.5 and 2 μg ml^{-1}, respectively.

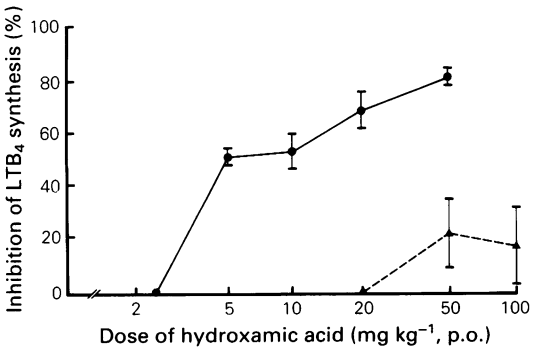


Figure 6 Effect of the acetohydroxamic acid BW A4C and a related hydroxamic acid BW A509C on the *ex vivo* synthesis of leukotriene B_4 (LTB_4) in rat whole blood stimulated with A23187. The inhibition of LTB_4 synthesis was monitored 3 h after oral administration of various doses of (●) BW A4C; (▲) BW A509C. The mean of data from 5 rats is plotted; vertical lines show s.e. mean.

The reproducibility of the assays for BW A137C and BW A797C were similar. An illustration of a chromatogram obtained 6 h after oral administration of BW A4C (50 mg kg^{-1}) to a rat is shown in Figure 7.

A comparison of the plasma concentrations of unchanged BW A4C after intravenous (2 mg kg^{-1}) and oral administration (50 mg kg^{-1}) is shown in Figure 8. The concentrations of BW A137C in plasma after 50 mg kg^{-1} p.o. were not as high nor was it detected for as long as BW A4C or BW A797C (Figure 8), which is consistent with biological data (Figure 5, Table 2).

Discussion

The novel acetohydroxamic acids BW A4C, BW A137C and BW A797C selectively inhibit the synthesis of LTB_4 and in this respect resemble nafazatrom and NDGA rather than the 'dual' 5-LO/CO inhibitor BW 755C. Since each of the novel compounds blocked metabolism of [^{14}C]-arachidonic acid to [^{14}C]-5-HETE, at concentrations close to those for inhibition of LTB_4 production in the same experiment (Table 1), their site of action is assumed to be the initial 5-LO reaction from which both 5-HETE

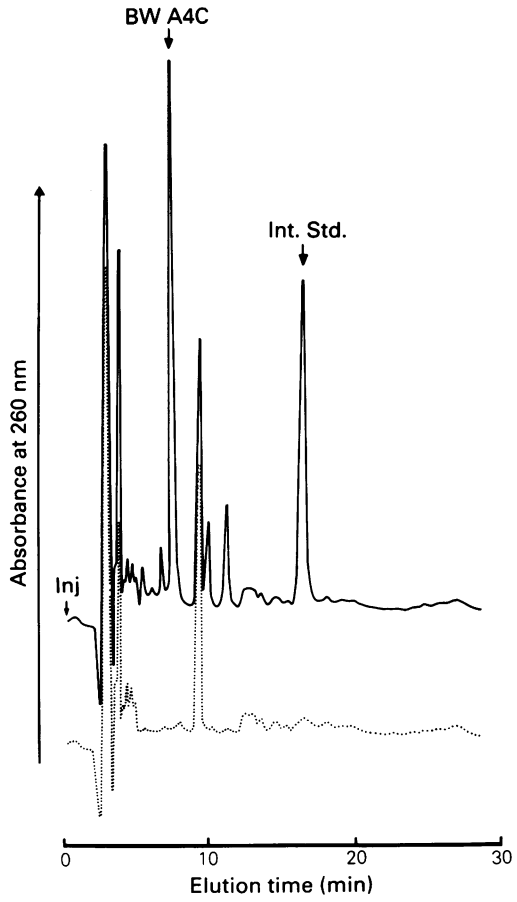


Figure 7 H.p.l.c. chromatogram of an extract of rat plasma obtained 6 h after administration of BW A4C (50 mg kg^{-1}, p.o.) (——). A chromatogram of an extract of untreated rat plasma is also illustrated (·····).

and LTB_4 are derived. However, as shown in Figure 3, a later Ca^{2+}-dependent step in the pathway of LTB_4 biosynthesis was also inhibited when 5-HPETE was used as substrate. The calcium-independent conversion of 5-HPETE to LTB_4 was unchanged. Similar effects have been observed with other inhibitors (Reynolds, 1986). Inhibition of the individual steps (LTA_4 synthase and LTA_4 hydrolase) has not yet been investigated, but the synthase is the more probable site of inhibition since it co-purifies with 5-LO and is Ca^{2+}-dependent (Rouzer *et al.*, 1986). Since it has been reported that the cytoplasmic 5-LO activity in human leucocytes is increased by a membrane-associated factor (Rouzer *et al.*, 1985), the activities of the acetohydroxamic acids were investigated using the cytoplasmic fraction as well as complete homogenates of human leucocytes. Results of such experiments (not shown) confirmed the stimulation of cytoplasmic LTB_4 production by the sedimentable fraction and showed that inhibition of LTB_4 synthesis by the acetohydroxamic acids was similar whichever enzyme preparation was used. This observation supports the view that the novel inhibitors act directly on the 5-LO and, possibly, LTA_4 synthase enzymes. Furthermore, LTB_4 synthesis was blocked with comparable potency whether it was measured in leucocyte-rich homogenates or in intact leucocytes stimulated with the ionophore

Table 2 Inhibition of leukotriene B_4-synthesis by acetohydroxamic acids *ex vivo* at various times after oral administration of acetohydroxamic acids

Compound	*ED_{50} values* (mg kg^{-1})*			
	1 h	3 h	6 h	10 h
BW A4C	1.8	6.6	9.0	30.2
BW A137C	11.6	22.5	44.5	Inactive at 100
BW A797C	3.9	8.5	7.2	Inactive at 50

* The data are presented as ED_{50} values (mg kg^{-1}) derived from regression analysis of 5-point log dose-response curves; each point is the mean obtained from 5 rats.

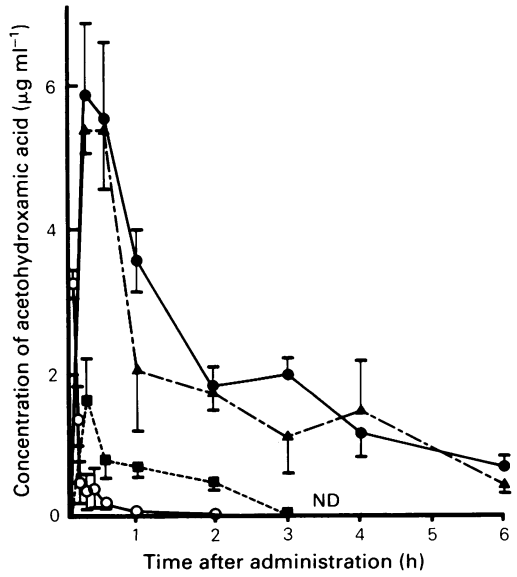


Figure 8 Concentrations of acetohydroxamic acids in plasma of rats dosed at various times before bleeding out. The mean of data from 5 rats at each time point is plotted; vertical lines show s.e. mean. ND = not detected (<0.2 μg kg^{-1}). (●) BW A4C, 50 mg kg^{-1}, p.o.; (○) BW A4C, 2 mg kg^{-1}, i.v.; (■) BW A137C, 50 mg kg^{-1}, p.o.; (▲) BW A797C, 50 mg kg^{-1} p.o.

A23187 (Table 1). Thus, there is no barrier to penetration of these lipophilic molecules across the plasma membrane.

Results of kinetic experiments have, so far, been inconclusive. The inhibition of LTB_4 synthesis by BW A4C was less at higher concentrations of arachidonic acid. However, the interpretation of this observation is not straightforward. The first reaction of the three-step sequence (arachidonic acid to LTB_4) was not monitored and initial rates of the 5-LO reaction cannot be measured satisfactorily in this preparation. Hence, we cannot draw the simple conclusion from this observation that the acetohydroxamic acid competes with substrate for the catalytic site of 5-LO. Nevertheless, it remains a possibility. Since the effect of the acetohydroxamic acids was lost by washing pretreated cells before challenge with A23187, it can be concluded that they are reversible inhibitors of 5-LO and do not interact covalently with the enzyme. However, the possibility of irreversible interaction with an activated form of the enzyme, in the presence of substrate and/or calcium, cannot be ruled out. It is possible that acetohydroxamic acids bind to non-haem iron in leucocyte 5-LO, as proposed by Corey *et al.* (1984) for arachidonic hydroxamates. However, there was no direct evidence that the acetohydroxamic acids described in this paper act by this mechanism. An alternative possibility is that the compounds inhibit 5-LO by virtue of the hydroxamic acid moiety being a reducing agent for one-electron reactions. Other lipoxygenase inhibitors such as NDGA, BW 755C and aminophenols (Thody *et al.*, 1987) appear to be antioxidants and may inhibit either enzyme activation (by peroxide or other factors, see Rouzer & Samuelsson, 1985) or catalysis. The selectivity for 5-LO may be conferred by the hydrophobic region of each acetohydroxamic acid acting as a substrate mimetic at the catalytic site of the enzyme.

The potency of each acetohydroxamic acid as an inhibitor of 5-LO was similar when the compounds were tested in human leucocyte-rich homogenates incubated with arachidonic acid or in blood cells stimulated with A23187. In contrast, the potencies of these compounds as inhibitors of TXB_2 synthesis were much higher in human leucocyte-rich homogenates than in whole blood. It is possible that this difference in inhibitor potency reflects a difference between the concentration of arachidonic acid added to homogenates and that mobilised endogenously by adding A23187 to intact cells. However, this seems unlikely since this also should have influenced the potency of the compounds as inhibitors of LTB_4 synthesis, but this was not observed. At present, therefore, the precise reason for this difference is unknown.

There has been a long-standing interest in the development of either selective 5-LO or 'dual' 5-LO/CO inhibitors as either anti-asthmatic or anti-inflammatory compounds. However, it must be emphasised that the hypothesis that 5-LO products (e.g. leukotrienes) are important in these disease states has not yet been substantiated. This is partly because many of the available inhibitors of 5-LO either suffer from problems of toxicity (e.g. BW 755C) or have *in vivo* activities that are too transient for their effects to be interpreted with confidence (e.g. nafazatrom, NDGA; McMillan *et al.*, 1986, Fuller *et al.*, 1987, and present data). Therefore, there is an urgent requirement for a selective 5-LO inhibitor that is non-toxic and has persistent activity *in vivo*. The present series of acetohydroxamic acids has been identified as fulfilling, at least in part, this need. These compounds all preferentially inhibited synthesis of the 5-LO product, LTB_4, in A23187-stimulated rat blood *in vitro*. Their potency in blood is similar to that in isolated leucocytes, suggesting either that these compounds are not bound to plasma proteins or that the affinity of the binding is low.

After either intravenous or oral administration, the degree of inhibition of 5-LO in rat blood *ex vivo* correlated well with the concentration of unchanged compound in plasma measured by h.p.l.c. For example, the relatively short duration of inhibition of 5-LO exhibited by BW A137C was shown to be due to the fact that lower plasma levels were reached than with BW A4C and BW A797C and the concentration decreased more rapidly. This provides confirmation of the validity of using the synthesis of LTB_4 in A23187-stimulated blood *ex vivo* as an indicator of blood levels. These data also suggest that the inhibitory activity is due to the unchanged compound and not to active metabolites.

Thromboxane B_2 synthesis in A23187-stimulated blood was not affected significantly after oral administration of the acetohydroxamic acids, which confirms the selectivity of the inhibition observed in rat blood *in vitro*. The plasma concentrations of unchanged acetohydroxamic acid, after oral administration, only transiently reached levels which would have been expected to reduce TXB_2 synthesis based on *in vitro* data. Therefore, the data obtained *in vitro* and *in vivo* are compatible.

It should be noted that the h.p.l.c. assay described here is not sufficiently sensitive to detect low concentrations of the acetohydroxamic acids which are still biologically active (i.e. the minimum detectable concentration of BW A4C and BW A797C is $0.1\,\mu g\,ml^{-1}$, and these concentrations cause more than 50% inhibition of LTB_4 synthesis in A23187-stimulated blood). Therefore, assessing the effect of compounds on LTB_4 synthesis *ex vivo* is a very useful and practical method of monitoring the

bioavailability, duration of activity and selectivity of potential 5-LO inhibitors. Using this technique we have established that the acetohydroxamic acids BW A4C, BW A137C and BW A797C inhibit 5-LO after both intravenous and oral administration; the onset of the inhibition after oral dosing is rapid and relatively persistent. A related hydroxamic acid BW A509C was far less potent and persistent in this model, although it inhibits LTB_4 synthesis *in vitro* with a similar IC_{50} to that of BW A4C (data not shown). These data suggest that hydroxamic acids exemplified by BW A509C are poorly absorbed and/or rapidly metabolised compared to the acetohydroxamic acids.

The evidence reported above shows that the acetohydroxamic acids described exert strong inhibition of 5-LO for 6 h after oral administration. Therefore, these compounds can be used to block the synthesis of 5-LO throughout the entire time course of the responses in animals subjected to acute inflammatory or allergic stimuli. Since the acetohydroxamic acids have only weak effects on CO, it will be necessary to use them in combination with a selective inhibitor of CO (e.g. indomethacin) in experiments where it is desirable to inhibit both routes of arachidonate oxidation. The effects of the acetohydroxamic acids alone or in combination with indomethacin on bronchoconstriction occurring in antigen-challenged guinea-pigs and on acute inflammatory responses in rats are described in accompanying papers (Payne *et al.*, 1988; Higgs *et al.*, 1988).

We wish to thank Messrs D. Wilson, C. O'Neil, A. Padfield, Ms A. Wallace, Mrs A. Petrovic and Mrs M. Swarup for their excellent technical assistance.

References

BORGEAT, P. & SAMUELSSON, B. (1979). Arachidonic acid metabolism in polymorphonuclear leukocytes: Effects of ionophore A23187. *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 2148–2152.

BRAY, M.A. (1986). Leukotrienes in inflammation. *Agents & Actions*, **19**, 87–99.

COREY, E.J., CASHMAN, J.R., KANTNER, S.S. & WRIGHT, S.W. (1984). Rationally designed, potent competitive inhibitors of leukotriene biosynthesis. *J. Am. Chem. Soc.*, **106**, 1503–1504.

FULLER, R.W., MALTBY, N., RICHMOND, R., DOLLERY, C.T., TAYLOR, G.W., RITTER, W. & PHILIPP, E. (1987). Oral nafazatrom in man: effect on inhaled antigen challenge. *Br. J. Clin. Pharmacol.*, **23**, 677–681.

GRESELE, P., ARNOUT, J., COENE, M.C., DECKMYN, H. & VERMYLEN, J. (1986). Leukotriene B_4 production by stimulated whole blood; comparative studies with isolated polymorphonuclear cells. *Biochem. Biophys. Res. Commun.*, **137**, 334–342.

HAMBERG, M. (1976). On the formation of thromboxane B_2 and 12 L-hydroxy-5,8,10,14-eicosatetraenoic acid (12 ho-20:4) in tissues from the guinea-pig. *Biochim. Biophys. Acta*, **431**, 651–654.

HAMBERG, M. & SAMUELSSON, B. (1974). Prostaglandin endoperoxides. Novel transformation of arachidonic acid in human platelets. *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 3400–3404.

HIGGS, G.A., FOLLENFANT, R.L. & GARLAND, L.G. (1988). Selective inhibition of arachidonate 5-lipoxygenase by novel acetohydroxamic acids: effects on acute inflammatory responses. *Br. J. Pharmacol.*, **94**, 547–551.

HIGGS, G.A., MONCADA, S. & VANE, J.R. (1984). Eicosanoids in inflammation. *Ann. Clin. Res.*, **16**, 287–299.

JACKSON, W.P., ISLIP, P.J., KNEEN, G., PUGH, A. & WATES, P.J. (1988). Acetohydroxamic acids as potent, selective, orally active 5-lipoxygenase inhibitors. *J. Med. Chem.*, **31**, 499–500.

LEWIS, R.A. (1985). A presumptive role for leukotrienes in obstructive airways diseases. *Chest*, **88**, 98S–102S.

MARDIN, M. & BUSSE, W.D. (1983). Effect of nafazatrom on the lipoxygenase pathways in PMN leukocytes and RBL-1 cells. In *Leukotrienes and other Lipoxygenase Products*. ed. Piper, P.J. pp. 263–274 Chichester: Research Studies Press.

McMILLAN, R.M., MILLEST, A.J., PROUDMAN, K.E. & TAYLOR, K.B. (1986). Evaluation of lipoxygenase inhibitors *ex vivo*. *Br. J. Pharmacol.*, **87**, 53P.

PALMER, R.M.J., STEPNEY, R.J., HIGGS, G.A. & EAKINS, K.E. (1980). Chemokinetic activity of arachidonic acid lipoxygenase products on leucocytes of different species. *Prostaglandins*, **20**, 411–418.

PAYNE, A.N., GARLAND, L.G., LEES, I.W. & SALMON, J.A. (1988). Selective inhibition of arachidonate 5-lipoxygenase by novel acetohydroxamic acids: effects on bronchial anaphylaxis in anaesthetized guinea-pigs. *Br. J. Pharmacol.*, **94**, 540–546.

RANDALL, R.W., EAKINS, K.E., HIGGS, G.A., SALMON, J.A. & TATESON, J.E. (1980). Inhibition of arachidonic acid cyclo-oxygenase and lipoxygenase activities of leucocytes by indomethacin and compound BW 755C. *Agents & Actions*, **10**, 553–555.

REYNOLDS, C.H. (1986). Ca^{2+} requirements in the pathway of leukotriene B_4 biosynthesis. *Biochem. Soc. Trans.*, **14**, 1049–1050.

ROUZER, C.A., MATSUMOTO, T. & SAMUELSSON, B. (1986). Single protein from human leucocytes possesses 5-lipoxygenase and leukotriene A_4 synthase activities. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 857–861.

ROUZER, C.A. & SAMUELSSON, B. (1985). On the nature of the 5-lipoxygenase reaction in human leucocytes: Enzyme purification and requirement for multiple stimulatory factors. *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 6040–6044.

ROUZER, C.A., SHIMUZU, T. & SAMUELSSON, B. (1985). On the nature of the 5-lipoxygenase reaction in human leucocytes: Characterisation of a membrane-associated stimulatory factor. *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 7505–7509.

SAMUELSSON, B. (1983). Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. *Science*, **220**, 568–575.

SALMON, J.A. (1978). A radioimmunoassay for 6-keto-prostaglandin $F_{1\alpha}$. *Prostaglandins*, **15**, 383–397.

SALMON, J.A. (1986). Inhibition of prostaglandin, thromboxane and leukotriene biosynthesis. In *Advances in Drug Research*, Vol. 15 ed. Testa, B. pp. 111–167. London: Academic Press.

SALMON, J.A., SIMMONS, P.M. & PALMER, R.M.J. (1982). A radioimmunoassay for leukotriene B_4. *Prostaglandins*, **24**, 225–235.

THODY, V.E., BUCKLE, D.R. & FOSTER, K.A. (1987). Studies on the anti-oxidant activity of 5-lipoxygenase inhibitors. *Biochem. Soc. Trans.*, **15**, 416–417.

(Received June 15, 1987
Revised November 15, 1987
Accepted January 28, 1988)