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PHOSPHOLIPASE A_2 INHIBITION BY ALKYLBENZOYLACRYLIC ACIDS

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Abstract—3-(4-Alkylbenzoyl)acrylic acids (ABAAs) were synthesized by acylation of alkylbenzenes with maleic anhydride and then screened *in vitro* for inhibition of phospholipase A_2 (PLA_2) from snake venom and from porcine pancreas. The inhibitory potency of ABAAs increased with the length of the alkyl residues resulting in IC_{50} values of between 10^{-7} and 10^{-4} mol/L. The most potent inhibitors of the snake venom PLA_2 were the 4-(*n*)-hexadecyl and octadecyl (OBAA) derivatives. Kinetic experiments referred to a time-dependent inhibitory reaction. Irreversibility was examined by dilution and dialysis. A molar ratio of inactivation of OBAA of nearly 20 was estimated. Double reciprocal replots of the apparent inactivation constants to the concentration of OBAA gave a (pseudo) first order rate constant of inactivation of 2.3 min^{-1}. For the dissociation constant of the enzyme–inhibitor intermediate, a value of 6×10^{-6} mol/L was obtained. On the other hand, the PLA_2 from porcine pancreas seemed hardly to be inhibited by ABAAs. The present data are discussed in relation to the proposed model for PLA_2 inactivation by manoalide. In human PMNs leukotriene B_4 and 5-HETE production was essentially reduced. In human platelets the thrombin-induced TxA_2 production was reduced. Since these effects disappeared after addition of arachidonic acid, these findings refer to a PLA_2 inhibition. The immunologically induced bronchospasm in guinea pigs was significantly and dose-dependently inhibited by OBAA. This indicates that ABAAs might be useful in treating allergic diseases, such as asthma, eczema, allergic shock and others.

Phospholipase (PLA_2‡) is a key enzyme in the release of unsaturated fatty acids from phospholipids and is therefore of outstanding importance for the synthesis of eicosanoids which might be involved in pathophysiological states such as inflammation, asthma, eczema and others. This makes PLA_2 an attractive target for new inhibitory agents. Most of the non-steroidal PLA_2 inhibitors reported so far are only poorly active or they are rather unsuitable for biochemical or clinical application, e.g. the phospholipid substrate-like PLA_2 inhibitors. Moreover, for many compounds inhibitory potency has been shown to be strongly dependent on the physicochemical structure of the substrate phospholipids used. To minimize such surface-related influences the use of the substrate in the form of mixed micelles might be advantageous.

Generally, PLA_2 activity is more effectively suppressed by active site-directed inhibitors, for instance halomethylketones like 4-bromophenacylbromide (BPB), but these compounds are relatively non-selective and rather toxic. For a couple of years the natural marine sponge product, manoalide, has been considered to be an interesting antiphospholipase agent because of its effectiveness *in vivo* [1, 2].

The present paper deals with a new group of aroylacrylic acids that were found to be potent irreversible inhibitors of PLA_2 from snake venom. However, they are apparently not inhibitory against porcine pancreas PLA_2. In addition, we determined the influence of these new substances on the formation of leukotriene B_4 (LTB_4) and 5-HETE by human polymorphonuclear leukocytes (PMN) and on the production of thromboxane A_2 (TxA_2) by human platelets. These investigations also strongly suggest that the reduction of eicosanoid generation is due to the PLA_2 inhibition.

MATERIALS AND METHODS

Materials. Chemical substances used were of analytical grade. PLA_2 prepared from crude snake venom of *Vipera russelli* (Gettel, Woltersdorf, Germany) was purified to 290 U/mg (determined by the use of the mixed micellar substrate, see below). Purity was examined by SDS–PAGE Porcine pancreas PLA_2 (600 U/mg, Boehringer, Mannheim, Germany) was freed from ammonium sulphate by dialysis. Lipoxygenase (LOX) from soybean (Serva, Heidelberg, Germany, 40 U/mg, polarographic assay) was applied without further purification. Linoleate (Sigma Chemical Co., St Louis, MO, U.S.A., 90–95%; or Merck, Darmstadt, Germany, 99%) was used as potassium salt for the LOX assay. The very simple synthesis of BPB was performed

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‡ Abbreviations: ABAAs, 3-(4-alkylbenzoyl)acrylic acids; BPB, 4-bromophenacylbromide; HBAA, 3-(4-hexadecylbenzoyl)acrylic acid; LOX, lipoxygenase, OBAA, 3-(4-octadecylbenzoyl)acrylic acid; PGE_2, prostaglandin E2; PGS, PG synthase; PLA_2, phospholipase A_2; PMN, polymorphonuclear leukocytes; LTB_4, leukotriene B_4; PBS, phosphate-buffered saline; TxH_2, thromboxane A_2; PAF, platelet-activating factor; TFA, trifluoroacetic acid; AA, arachidonic acid.

according to the literature (textbook knowledge). It was purified by 3-fold recrystallization in ethanol.

Synthesis of inhibitors. An aliquot of 0.25 mol of anhydrous $AlCl_3$ in 80 mL freshly distilled nitrobenzene was added in small portions to a stirred solution of 0.1 mol alkylbenzene and 0.11 mol maleic anhydride which was freshly recrystallized from ether.

Temperature was kept between 40 and 50°. After 1 hr of stirring the reaction was allowed to cool to room temperature, followed by stirring for 4 hr. After standing overnight the reaction mixture was decomposed with 500 g of ice and 30 mL of concentrated hydrochloric acid. The solvent was removed by steam distillation. The subsequent layers were separated and the organic layer was handled with *n*-hexane or *n*-heptane leading to crystallization. Further purification of 3-(4-alkylbenzoyl)acrylic acids (ABAAs) was realised by 2-fold recrystallization from *n*-heptane.

Analytical data. ^{1}H-NMR spectra were recorded on a Bruker WP 200 instrument. For the monitoring of mass spectra a VG analytical ZAB-HSQ apparatus was used. The elementary analysis of all compounds was within ±0.4% of theory.

OBAA: ^{1}H-NMR ($CDCl_3$): $\delta = 0.85$ ppm (3H,t,J = 13 Hz), $\delta = 1.23$ ppm (30 H, s), $\delta = 2.66$ ppm (2H,t, J = 15 Hz), $\delta = 6.86$ ppm (1 H,d,J = 16 Hz,CH = CH), $\delta = 7.58$ ppm (4H,m, Ar-H), $\delta = 7.98$ ppm (1 H,d,J=16 Hz,CH = CH), MS *m/e*: 428 (M^+), m.p.: 89–90°.

PLA_2 in vitro assays. Changes in snake venom PLA_2 activity were monitored by the use of a coupled enzymatic assay with soybean-15-lipoxygenase as described by Köhler *et al.* [3] and by Wallach and Brown [4], respectively.

Additionally, sodium cholate (6.7 μmol/L) was added. Partial synthetic 1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine (90 μmol/L, 90–95% of free acid as linoleic acid) was prepared according to Ref. 3 and applied in the form of mixed micelles with Triton X-100 in a molar ratio of 1:4. The decrease in oxygen content was recorded continuously. Initial slopes or accumulated products during the first 40 sec were used for kinetic analysis.

Inhibition of porcine pancreatic PLA_2 was examined by recording the slopes resulting from pH change. For determination the decrease in 0.05 pH units (from pH 7.90 to 7.85) was utilized after starting the reaction with chromatographically purified egg yolk phosphatidylcholine–Triton X-100–sodium dodecyl sulphate 1–2–0.4 molar, phospholipid content 1 mmol/L as the substrate.

Determination of the molar ratio of inactivation. Purified *V. russelli* PLA_2 (13.4 μg, 4.48 μmol/L) was incubated in small polyethylene beakers at 20° for 24 hr. Tris–HCl 0.1 mmol/L, pH 8.5 in a total volume of 200 μL Ca^{2+} 0.1 mol/L and inhibitor (5–100 μmol/L) were added. The product level after 20 sec compared with the medium value of two probes without inhibition was used for determination of residual activity. Protein content was measured by the method of Lowry *et al.* [5] with human serum albumin as standard or by the biuret method [6] and by a turbidimetric determination with bovine serum albumin as standard as prescribed in Ref. 7. To separate from smaller peptides, salts and sugar, use was made of a precipitation procedure with trichloroacetic acid according to Ref. 8.

Dialysis. Dialysis was carried out against Tris–HCl 0.1 mol/L, pH 8.5, at room temperature for 7 hr by shaking the dialysis cell quickly. A flow rate of 0.8 L/hr was maintained. Snake venom PLA_2 (2.24 μmol/L) and inhibitor (200 μmol/L) were incubated together in the absence of Ca^{2+}. At the beginning of the experiment the remaining enzyme activity was 4.5% of control without inhibitor.

Prostaglandin synthase (PGS) activity of sheep seminal vesicles. A microsomal preparation of sheep seminal vesicles was used for testing the inhibition of PGS. Inhibition was measured by using the polarographic method described for LOX.

Conditions: 1.6 mg protein (Lowry), arachidonic acid (free acid): 78 μmol/L, tryptophan: 2.7 mmol/L, haemin: 2.6 μmol/L, Tris–HCl: 50 mmol/L, pH 8.0, 30°; preincubation with the inhibitor: 5 min.

Materials for determination of LTB_4 and 5-HETE generated by human PMN. Heparin sodium without conservating agents was purchased from Gedeon Richter Budapest, Hungary; arachidonic acid (AA) (99%) from Sigma; calcium ionophore A 23187 (free acid) from Boehringer; Macrodex® 6% solution from Schiwa (Glandorf, Germany); prednisolone from Jenapharm GmbH (Jena, Germany) LTB_4 and 5-HETE were gifts of F.-P. Gaede, Inst. of Pharmacy, Univ. of Tübingen (Germany). BN 50730 was a gift from Dr P. Braquet, Institut Henri Beaufor (Le Plessis Robinson, France).

Preparation and stimulation of human PMN. Human PMN were isolated as follows: heparinized venous blood from healthy volunteers free of medication was mixed with 0.2 vol. of 6% dextran 75 (Macrodex® 6%) and allowed to stand for 20 min at 37°. The leukocyte-rich supernatant was layered onto a Ficoll-Paque gradient (1.077 g/cm^3; Pharmacia, Uppsala, Sweden). After centrifugation (400 *g*, 30 min, room temp.), the pellet was washed with phosphate-buffered saline (PBS), pH 7.4. Remaining erythrocytes were lysed in distilled water. After a further washing step in PBS the cells were resuspended in HBS. The 5×10^6 cells suspended in 1 mL HBS were incubated with the test compounds or with solvent alone for 5 min at 37°. Ethanol (max. final concn 0.5%, v/v) was used as a solvent. After stimulating the cells with the calcium ionophore A 23187 (5 μM) for 5 min, the reaction was stopped by adding 1 mL of cold methanol and 5 μL of acetic acid.

Extraction and analysis of lipoxygenase (LOX) products. The methods of Luderer *et al.* [9] and Steinhilber *et al.* [10] were modified as follows: After centrifugation, the supernatants containing 25% (v/v) methanol were passed through Bakerbond SPE C_{18} cartridges (J. T. Baker), which had been conditioned with 5 mL of methanol, 5 mL of distilled water and 5 mL of 25% (v/v) methanol. After washing the sample with 2 mL of water and 2 mL of 25% (v/v) methanol, eicosanoids were eluted with 2 mL of methanol. After evaporation of the solvent under nitrogen and reconstitution in 100 μL of methanol, 40 μL of the sample were injected onto a Radial Pak HPLC-cartridge (100 × 5 mm i.d.) with

Novopak C_{18} (4 μm particle size). HPLC was performed with two Waters 510 pumps connected with a Waters automated gradient controller 680. In order to elute all compounds of interest in a single run, a stepwise gradient from MeOH/H_2O/trifluoroacetic acid (TFA) 60/40/0.008 (v/v/v) to MeOH/TFA 100/0.008 (v/v) over 29 min was performed. UV absorbance was monitored at 270 nm for LTB_4 and 235 nm for 5-HETE. Identification of the 5-LOX products was performed by coinjection with authentic standards and their total suppression by potent LOX inhibitors.

Preparation of washed human platelets. Platelet-rich plasma was prepared by centrifugation of 10 mL EDTA-buffered blood (15 min, 200 *g*, room temp.). The platelet-rich plasma was diluted with PBS, pH 6.0, and centrifuged at 2000 *g* for 10 min. After an additional washing step, the resulting platelet pellet was resuspended in 1 mL PBS, pH 7.4, with 0.1% glucose.

Incubation of platelets and extraction of the AA metabolites. Cell suspension (1 mL, 2.5–5 × 10^8 cells) was incubated at 37° for 3 min in the presence of 3-(4-hexadecylbenzoyl)acrylic acid (HBAA) or the solvent alone (ethanol, final concn 1%, v/v). The cells were stimulated with thrombin (5 U/mL). After 5 min the reaction was stopped by addition of 1 mL of cold methanol.

The sample was spiked with 16,16-dimethyl-PGE_2 (1 μg) as an internal standard, diluted with 2 mL of ice-cold water and acidified with 1 N HCl to pH 3. The mixture was centrifuged at 2000 *g* for 10 min. The supernatant was extracted according to the method of Luderer *et al.* [9], modified to meet the present requirements. Baker C-18 disposable columns were first preconditioned with 2 mL of methanol and 2 mL of PBS buffer, pH 3. Then the supernatants were loaded on the column. Before elution with 2 mL of methanol, the column was washed with 2 mL of 25% (v/v) methanol, 2 mL of water and 2 mL of *n*-hexane.

The eluate was evaporated to dryness under a stream of nitrogen at 37°. The residue was reconstituted in 100 μL of methanol.

Derivatization procedure. Following the method of Knospe *et al.* [11], the arachidonic acid metabolites were derivatized to the corresponding anilides, which show a strong electrochemical response. As reagents were used 2,4-dimethoxyaniline-HCl and 1-ethyl-3-dimethylaminopropylcarbodiimide-HCl.

HPLC analysis. HPLC separations were carried out using a Radial-Pak cartridge (100 × 5 mm i.d.) with Novapak C_{18} (4 μm particle size). The HPLC system consisted of a Waters 590 pump, a Waters U 6 K injector and a Waters 460 electrochemical detector, equipped with a thin-layer glassy carbon electrode. The eluent was MeOH/H_2O 65/35 (v/v), containing 5 mM lithium perchlorate and 0.45 mM TFA. The flow was 1 mL/min. The potential of the Waters 460 electrochemical detector was set to +1.10 V versus a silver/silver chloride electrode (filling: 3 M lithium chloride in 65% aqueous methanol).

Allergic bronchospasm in passively sensitized guinea pigs. Male guinea pigs (Berger, Langburkersdorf, Germany), body wt 350–500 *g*, were passively sensitized by i.v. injection into the dorsal penis vein of 1.5–6 mL/kg (depending on the antibody titer) of rabbit antiovalbumin serum. Challenge was performed 24 hr later by i.v. injection of 1 mg ovalbumin in isotonic saline. Thereafter, the trachea of the guinea pigs was canulated under ethylurethane anaesthesia with muscle relaxation (1.5 g/kg ethylurethane plus 2 mg/kg pancuronium bromide i.p.). The guinea pigs were artificially ventilated with a pump (1.5 mL × 70/min; active insufflation and passive expiration) and the respiratory pressure was measured with a y · t recorder [12].

Table 1. IC_{50} values for 3-(4-alkylbenzoyl)acrylic acids with PLA_2 from *V. russelli*

(*n*)-Alkyl substituent*	IC_{50} value (μmol/L)
Ethyl	<500
Butyl	200
Pentyl	70
Hexyl	37
Octyl	9.8
Dodecyl	1.3
Tetradecyl	0.2
Hexadecyl	0.14
Octadecyl (OBAA)	0.07

* Yield (%) was: 39 (ethyl); 35 (butyl); 28 (pentyl); 45 (hexyl); 42 (octyl); 48 (dodecyl); 50 (tetradecyl); 58 (hexadecyl); 56 (octadecyl = OBAA).

For assay conditions see Material and Methods.

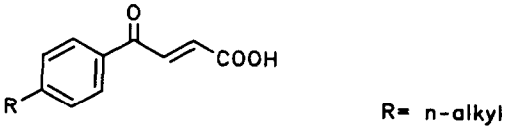
RESULTS

Determination of PLA_2 inhibition

A potent inhibition of PLA_2 from *V. russelli* was obtained with the 3-(4-alkylaroyl)acrylic acids (see Structure 1).

The IC_{50} values determined with snake venom PLA_2 were shown to be strongly dependent on the chain length of the arene's para substituent (Table 1).

For 3-(4-phenylbenzoyl)acrylic acid and for 3-(4-octyloxynaphthoyl)acrylic acid IC_{50} values of 86 and 6.8 μmol/L were found, respectively. Assuming a length of about four carbons for the diameter of the phenyl residue and nine methylene groups for the octyloxy chain, a graph of IC_{50} as a function of the number of CH_2 groups fits both these substances as well as ABAAs (Fig. 1). An IC_{50} of greater than



Structure 1.

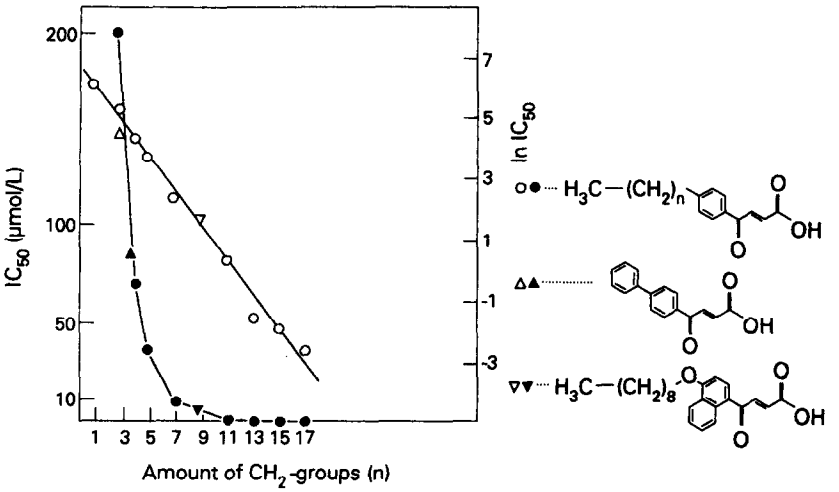


Fig. 1. Dependence of inhibitory activities (IC_{50}) of substituted aroylacrylic acids against PLA_2 on the chain length of (n)-alkyl residues. Preincubation in the presence of 0.01 mol/L Ca^{2+} for 3 min at 37°, pH 8.5; PLA_2 content 1.7×10^{-8} mol/L. (●) Numeric and (○) semilogarithmic plot.

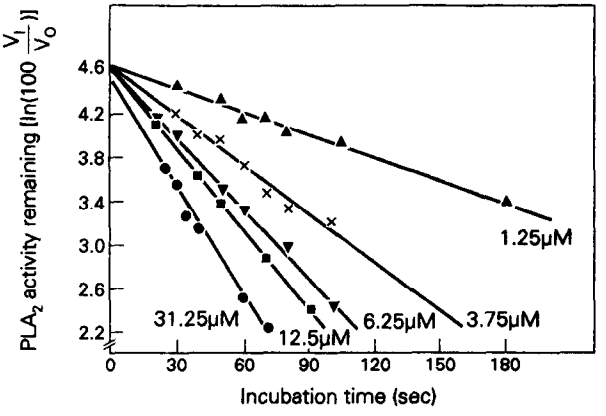


Fig. 2. Inactivation of PLA_2 (*V. russelli*) by OBAA. Preincubation of the enzyme (3.4×10^{-8} mol/L) with inhibitor at 37°, pH 8.5, in the presence of 0.01 mol/L Ca^{2+}. Assay conditions as described under Materials and Methods. V_0, Initial rate of reaction without inhibitor; v_i, initial rate of reaction with inhibitor.

500 μmol/L was obtained for 3-(4-bromobenzoyl)-acrylic acid.

Kinetic analysis. A more detailed investigation of the most inhibitory derivative, the 3-(4-octadecylbenzoyl)acrylic acid (OBAA), yielded evidence for inactivation proceeding in a time-dependent manner (Fig. 2). From the replot of slopes (Fig. 3) the kinetic parameters of inactivation were found with 2.3 min^{-1} for the first order rate constant and 5.9 μmol/L for the dissociation constant of the enzyme–inhibitor complex.

Dilution tests and dialysis experiments. Both tests supported the idea of an irreversible mode of inactivation. A 10-fold dilution of an incubation probe examined at two different time intervals (at 65% and 85% inhibition) resulted in negligible

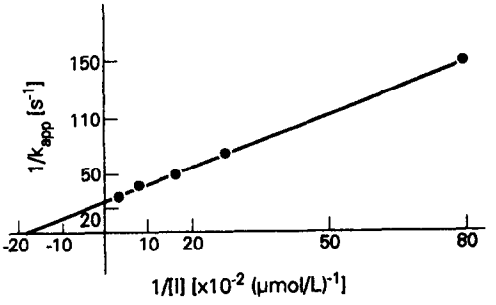


Fig. 3. Replot of the inactivation constants from slopes. For estimation of kinetic parameters the slopes given in Fig. 2 were plotted in dependence on the applied concentration of OBAA.

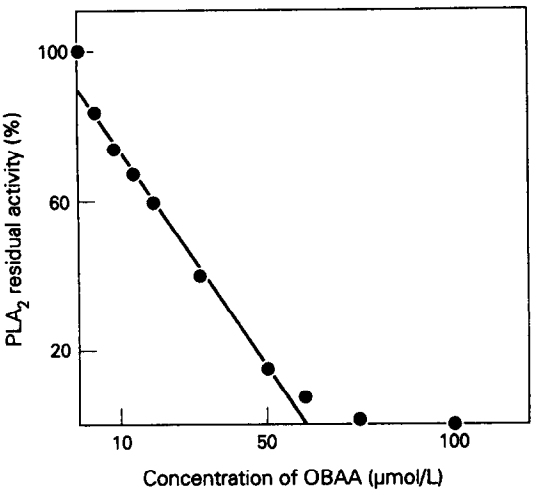


Fig. 4. Titration of PLA_2 (*V. russelli*) with OBAA. For each point 4.48 μmol/L PLA_2 (3.9 U) were incubated with inhibitor in 200 μL of 0.1 mol/L Tris–HCl, pH 8.5, 0.01 mol/L Ca^{2+}, for 36 hr at 20°. Remaining activities had been corrected for diminution of PLA_2 activity during the incubation period.

inhibition changes of less than 5%. No reconstitution of PLA_2 activity was reached after 5 hr of dialysis.

Molar ratio of inactivation. In further experiments the molar ratio of total inactivation of the used snake venom PLA_2 was estimated. Figure 4 shows that nearly 20 mol of OBAA are required to block 1 mol of the enzyme by 100%. The exact value of 17.9 mol of OBAA/mol PLA_2 was determined by linear regression analysis (quotient of 80.2 μmol/L OBAA to 4.48 μmol/L PLA_2). This ratio is based on the estimation of protein content by the method of Lowry *et al.* [5] and by the biuret method. The protein band of the purified enzyme detected after SDS–PAGE (Fig. 5) is regarded as being a phospholipase of the A_2 type. According to Ref. 13 a M_r of the purified enzyme of 15,000 was calculated.

Influence on porcine pancreas PLA_2. Strikingly, no significant inhibition of porcine pancreas PLA_2 could be detected. In the absence of Ca^{2+}, no reduction of PLA_2 activity was measured after 3 hr 30 min at 37°, at 300 μmol/L of OBAA.

Inhibition of soybean-15-LOX. ABAAs show only weak inhibitory effects against soybean-15-LOX independent of their close structural relationship to fatty acids. IC_{50} values greater than 100 μmol/L were determined with the separate investigation of LOX activity. No inhibition was found below 1 μmol/L.

Inhibition of PGS. Investigation of OBAA and also of 3-(4-butylbenzoyl)acrylic acid and of 3-(4-tetradecylbenzoyl)acrylic acid showed no inhibition of sheep seminal vesicle PGS at 10^{-5}, 10^{-4} or 10^{-3} mol/L.

Influence on LTB_4 and 5-HETE release by human PMNs and on TxA_2 release by human platelets

The arachidonic acid released under PLA_2 catalysis is metabolized via two main pathways: the lipoxygenase and the cyclooxygenase pathways. A substrate shortage caused by PLA_2 inhibition should therefore suppress the production of both kinds of AA metabolite from endogenous substrate. We tested the effects of OBAA and of HBAA on LTB_4 and 5-HETE release from calcium ionophore A 23187-stimulated human PMN and the effect of HBAA on the thrombin-induced release of TxA_2 from human platelets.

OBAA and HBAA inhibited the endogenous production of LTB_4 and 5-HETE in A 23187-stimulated PMN in a concentration-dependent manner (Figs 6 and 7). The IC_{50} values of OBAA and HBAA are about 35 and 20 μM, respectively, for LTB_4 production and 20 and 2 μM, respectively, for the production of 5-HETE. 4-Bromophenacylbromide, as a known PLA_2 inhibitor, is also effective in this system and shows IC_{50} values of 7 μM for LTB_4 and 5 μM for 5-HETE.

Adding 25 μM of AA to the PMN suspension immediately before the LOX reaction was started by A 23187, the inhibitory effect of OBAA or HBAA disappeared (Figs 6 and 7). These results show that the limitations of AA as substrate provoke consequences for both the LOX and the COX pathway.

Influence on allergic bronchospasm

OBAA caused significant bronchospasmolysis in a dose-dependent manner between 2.5–7 mg/kg i.v. (Table 2). This is a relatively high potency in comparison with that of the reference compound oxyethyltheophylline, which caused the same effects only at 50–100 mg/kg [2]. The new platelet activating factor (PAF) antagonist BN 50730 produced a moderate inhibition of bronchospasm, also (Table 2). Combined administration of a low dose of OBAA (2.5 mg/kg) with BN 50730 had an additive effect (Table 2).

DISCUSSION

PLA_2 inhibition

A large body of compounds has been described as PLA_2 inhibitors, mainly substrate analogs and natural products, e.g. sesterterpenes such as luffariellolide and the above mentioned manoalide, the alkaloid aristocholic acid, and others [14]. Our first attempts to screen for potential PLA_2 inhibitors included screening analogous substrate phospholipids with ether bridges, cycloaliphatic, aromatic and -alkyl branched fatty acids, lyso-phospholipid derivatives, aryl fatty acids and keto-acid derivatives (data not shown). Among these substances the keto-acids and some of their semicarbazones caused a weak inhibition of PLA_2 activity. More important for the present paper seems to be the fact that no inhibitory potency for nearly all of these heterogenous compounds and no activating effects below 10 μmol/L could be detected (data not shown). This should be accepted as a positive argument with respect to the prevention of substrate-related inhibitory artefacts caused by pure interaction of lipophilic substances with the phospholipid surface. The utilization of a mixed micellar substrate allows the total suppression of troublesome lag phases

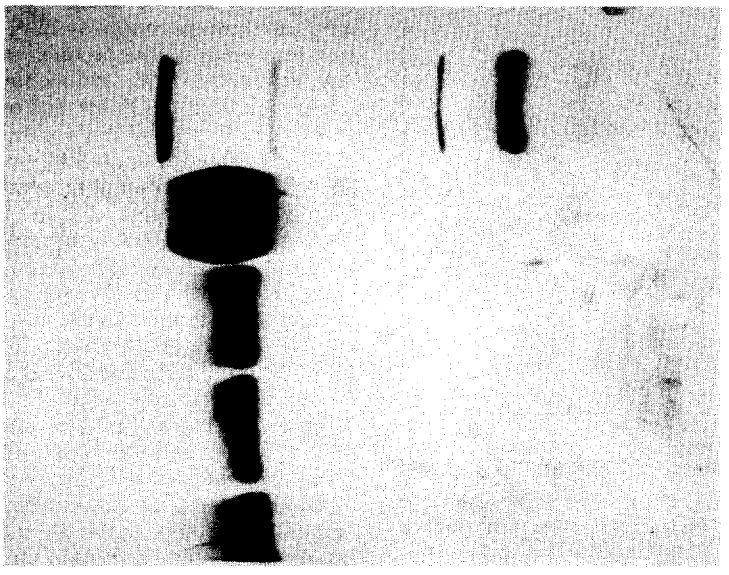


Fig. 5. SDS–PAGE of PLA_2 from *V. russelli*. Crude lyophilized toxin was dissolved in water and then freed from disturbing proteins and from salts by simple precipitation after incubation in boiled water for 3 min. Standards (M_r): 1, α-lactalbumin (14,400); 2, trypsin inhibitor (20,100); 3, carboanhydrase (30,000); 4, ovalbumin (43,000); 5, albumin (67,000).

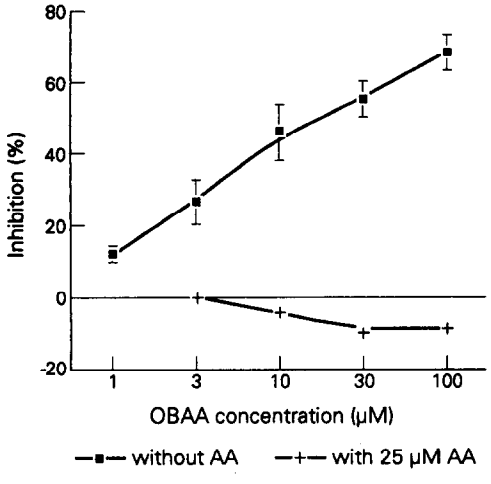


Fig. 6. Inhibition of 5-HETE production in A 23187-stimulated human PMN by OBAA (mean ± SD); three separate experiments. IC_{50} for LTB_4; 3.5×10^{-5} mol/L.

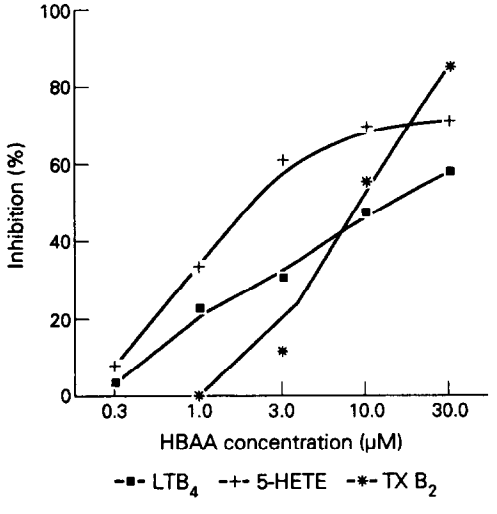


Fig. 7. Inhibition by HBAA of TxA_2 production induced by thrombin in washed human platelets (*) and reduction of 5-HETE (+) as well as of LTB_4 (■) generation in A 23187-stimulated human PMN; two separate experiments. All inhibiting effects completely disappeared after addition of 25 μM AA.

which hamper correct determination of initial velocities. Under the present assay conditions using isotropic substrates, Michaelis–Menten kinetics are reached at least for the initial part of reaction. For the applied snake venom PLA_2 assay the reaction ceases after 1 min apparently because of accumulation of unsaturated free fatty acid salts (product inhibition). If a short initial time interval is used for registration, product inhibition is without influence on the measured parameter.

Long chain substituted aroylacrylic acids show potent inhibition of snake venom PLA_2 from *V. russelli*. The straight line given by the semilogarithmic plot of IC_{50} against the number of methylene groups, which is well fitted by both long chain residues and simple planar aromatic substituents like the 4-phenyl

Table 2. Influence on allergen-induced bronchospasm in passively sensitized guinea pigs

Substance	Dose (mg/kg)	Inhibition (%) of bronchospasm with time (in min)		
		3	5	7
OBAA	2.5 i.v.*	20	23	22
OBAA	5.0 i.v.	67‡	68‡	68‡
OBAA	7.0 i.v.	83‡	83‡	87‡
BN 50730	10.0 i.v.*	48‡	51‡	58‡
OBAA+ BN 50730	2.5 + 10 i.v.*	55‡	62‡	74‡
Prednisolone	25.0 i.m.†	45‡	53‡	61‡

* Injection 15 min before challenge.
† Injection 18 hr before challenge.
‡ $P < 0.05$ (Student's t-test).
N = 5 animals per group.

residue, refers to a strong correlation between hydrophobicity and inhibitory potency. This is not in contradiction to structural models proposed for most kinds of PLA_2 since it has been well documented that the active center of PLA_2 is embedded in a hydrophobic core. The other two terms formulated for quantitative structure–activity relationships, the Hammett parameter and the steric parameter, seem to have only a negligible influence on the investigated ABAAs. A premise to this is unambiguously the existence of an unchanged vinylogic carbonyle. In other words, the benzoylacrylic acid structure is supposed to be the essential part for the irreversibility of the reaction, and the lipophilic moiety determines the effectiveness of binding. Thereby it seems remarkable that the snake venom PLA_2 used in our experiments shows considerably higher affinity to OBAA than to several long chain saturated fatty acids, 4-(*n*)-alkylbenzoic acids, 4-(*n*)-alkylcyclohexylcarbonic acids (alkyl = propyl- . . .dodecyl-) and -aryl branched fatty acids. So the most potent compound of this group tested, the 2-phenylpalmitic acid, shows a K_i value of 170 μmol/L. This refers to an affinity for OBAA exceeding the fatty acid derivatives by a factor of nearly 30.

Two results demonstrate the irreversible character of the modification step: (1) the time dependence of inactivation which follows first order kinetics and (2) the absence of reversibility studied directly by dialysis and dilution. Because of the good solubility of the free acids at pH 8.5, the dissociation of the enzyme–inhibitor complex should not be suppressed by the hydrophilic rinsing solution. The use of a basic buffer therefore avoids a misinterpretation which could be caused by a simple interaction of the hydrophobic inhibitor with the hydrophobic enzyme.

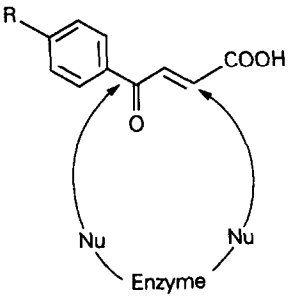
It should be mentioned that incubation was carried out in the presence of a saturating concentration of the cofactor Ca^{2+}. Ca^{2+} was found to protect the PLA_2 from inactivation by the active site-directed inhibitor BPB. With a (pseudo) first order rate constant of 2.3 min^{-1} the inactivation is more rapid by 50 times under these conditions than with the halomethylketone (data not given). The intersection of the abscissa shown in the secondary plot of $1/k_{app}$ against 1 (I) agrees well with the kinetic model of a Michaelis–Menten-type of inactivation. That means: a dissociable enzyme–inhibitor complex is built up before the dead-end complex is reached.

$$E + I \overset{K_I}{\rightarrow} E \,.\, y\, I \overset{k_{inact}}{\rightarrow} E\text{–}I$$

Though lacking the glycerol moiety of the phospholipid substrates, the approximated dissociation constant for the enzyme–inhibitor complex (K_I) of 6 μmol/L is one third of the medium apparent Michaelis constant (20 μmol/L) determined for this system.

In the presence of a molar excess of inhibitor by a factor of 1000, which is usually applied in the kinetic investigation of reversible inhibitors, the activity of PLA_2 from *V. russelli* toxin is totally suppressed by OBAA. This is in agreement with the potency of manoalide against bee venom PLA_2 published by Glaser *et al.* [15] but contradicts somewhat the only partial suppression of the cobra enzyme by manoalide and the related compound manoalogue described by Dennis and co-workers [16, 17]. Bennett *et al.* [18] and Glaser and Jacobs [19] gave evidence that PLA_2 from different sources may be more or less sensitive to manoalide dependent on the availability of essential lysine residues located at or near to the catalytic or substrate surface binding site. Particularly for the porcine pancreatic enzyme, a strongly reduced sensitivity towards manoalide inactivation has been reported.

A molar ratio of inactivation of nearly 1:1 could not be demonstrated. The relatively high molar ratio for OBAA of 17.9 compared to the published value for manoalide [15] may be caused in part by a time-dependent loss of inhibitory potency of OBAA. For the first 6 hr diminution is well fitted by first order kinetics, but then slows down. During this time period (40°, pH 8.5) 25–30% of inhibitory activity is lost. Additionally, for IC_{50}-estimation a molar ratio of 4 for OBAA resulted in the half maximum inhibition of the enzyme within 3 min of incubation time. The relation $1/v = f(I)$ (Dixon plot) gave exact straight lines for the highly reactive benzoylacrylic acid derivatives with tetradecyl, hexadecyl and



Scheme 1.

octadecyl residues only in the range of 0–80% of inhibition (and curves for the higher content of inhibitor to the abscissa and not to the ordinate as expected for such irreversible time dependent inhibitors), which contributes to the low ratio of inhibitor to enzyme present in these studies. Because of the fact that for total inactivation during longer incubation periods substantially higher levels of OBAA are required, side reactions of the sterically labile and chemically reactive inhibitor obviously cannot be excluded [20]. Unlike a detailed analysis of amino acids, the methods of protein content estimation used allow no determination of absolute values. To prevent an overestimation, a second run including a precepitation procedure to separate small peptides and short chain glycopeptides was utilized. As a result, no greater difference in protein content was found (PLA_2) fraction, before precipitation with trichloroacetic acid: 1.98 mg/mL, Lowry, 2.04 mg/mL, biuret determination; after precipitation: 1.92 mg/mL, Lowry. The much smaller value of 0.576 mg/mL (two determinations, difference 3%) found for the same protein preparation with the turbidimetric method according to Vera [7] was not used for further interpretation. With respect to the SDS–PAGE (Fig. 5), larger glycoprotein impurities—such a component with a M_r of 86,000 was described in Ref. 21—could also be excluded in the purified PLA_2 solution. Therefore, the low turbimetric value may not be caused by a greater degree of glycoprotein impurities, but may be related to protein specificities (particle size, breaking index) that complicate a simple comparison of *V. russelli* PLA_2 with the bovine serum albumin standard.

The reactive site of the inhibitor molecule is undoubtedly the keto-allylic structure. Nucleophiles like primary amines of lysine residues could attack the vinylogic carbonyle of alkylaroylacrylic acids with a subsequent covalent fixation via addition to α,β-unsaturated carbonyles. A carbonyle allylic structure is also present in the sesquiterpenes manoalide and luffariellolide. Unlike monoalide and the ABAAs, the inhibitions by luffariellolide and synthetic hydroxybutenolides [22, 23] were partially reversible. According to the postulated mechanisms of manoalide for the irreversible inactivation of PLA_2 [23], a two-step mechanism may be formulated for the ABAAs (see Scheme 1).

The dependence of the chain length of the 4-alkylphenyl residues on the inhibitory potency which we found agrees well with the influence of the hydrophobicity (trimethylcyclohexenyl moiety) on the inhibitory potencies of the manoalide analogues structures presented in Ref. 19.

Influence on 5-LOX activity

The amounts of LTB_4 and 5-HETE produced by stimulated PMN were essentially reduced by OBAA and HBAA. This effect is apparently due to substrate limitation. After addition of AA this inhibition was abolished (Figs 6 and 7). Therefore, the inhibition of the PMN 5-LOX as by OBAA or HBAA can be ruled out. This conclusion is confirmed by the finding that OBAA and HBAA are ineffective against soybean lipoxygenase.

Taking into account the strong effects of the alkylbenzoylacrylic acids, mainly of OBAA and HBAA against the snake venom enzyme, the PLA_2 is the most probable target enzyme for the inhibitory action of ABAAs in human PMN cells. The inhibition of TxA_2 release from platelets is interesting insofar as the phospholipase C is involved in the thrombin-induced activation of human platelets. A selective thromboxane synthase inhibition by ABAAs can be ruled out because the small amount of PGE_2 which is produced by human platelets after thrombin stimulation was inhibited in parallel to TxA_2. Otherwise, a greater amount of PGE_2 would be produced.

Inhibition of bronchospasm

The involvement of eicosanoids, mainly of leukotrienes and PAF, in asthma is very likely [24]. Since inhibition of PLA_2 cannot only decrease leukotriene formation but also PAF generation, the bronchospasmolytic activity of OBAA with the new PAF receptor antagonist BN 50730 could allow consideration of this combination as a useful therapy in bronchospasm and asthma [12]. The most promising therapy in asthma is anti-inflammatory [25, 26], which is the reason for the incomparable effectivity of the exogenous steroids in the late phase of asthma [26]. Although we could not measure an anti-inflammatory effect of OBAA in carrageenin rat paw edema (data not shown), such an activity might nevertheless be caused by the OBAA–BN 50730 combination in an allergic inflammation. Since the acute toxicity of OBAA in rats is moderate to low (data not shown) further investigations might be indicated and should be performed. In summary, we can state that ABAAs very likely reduce AA metabolism by an inhibition of PLA_2. They might be useful in the treatment of allergic diseases, e.g. asthma, eczema, anaphylactic shock, and others.

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