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Acetyl-11-keto-β-boswellic acid (AKBA): structure requirements for binding and 5-lipoxygenase inhibitory activity

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1 5-Lipoxygenase (5-LOX) products from endogenous arachidonic acid in ionophore-stimulated peritoneal polymorphonuclear leukocytes (PMNL) and from exogenous substrate (20 μM) in 105,000 *g* supernatants were measured.

2 The effects of natural pentacyclic triterpenes and their derivatives on 5-LOX activity were compared with the inhibitory action of acetyl-11-keto-β-boswellic acid (AKBA), which has been previously shown to inhibit the 5-LOX by a selective, enzyme-directed, non-redox and non-competitive mechanism.

3 The 5-LOX inhibitory potency of AKBA was only slightly diminished by deacetylation of the acetoxy group or reduction of the carboxyl function to alcohol in intact cells (IC_{50} = 1.5 vs. 3 and 4.5 μM, respectively) and in the cell-free system (8 vs. 20 and 45 μM).

5 β-Boswellic acid (β-BA), lacking the 11-keto function, inhibited 5-LOX only partially and incompletely, whereas the corresponding alcohol from β-BA, as well as amyrin, acetyl-11-keto-amyrin, 11-keto-β-boswellic acid methyl ester had no 5-LOX inhibitory activity up to 50 μM in either system.

5 β-BA only partially prevented the AKBA-induced 5-LOX inhibition, whereas the non-inhibitory compounds, amyrin and acetyl-11-keto-amyrin, almost totally antagonized the AKBA effect and shifted the concentration-inhibition curve for the incomplete inhibitor β-BA to the right. In contrast, the non-inhibitory 11-keto-β-BA methyl ester exerted no antagonizing effect.

6 The results demonstrate that the pentacyclic triterpene ring system is crucial for binding to the highly selective effector site, whereas functional groups (especially the 11-keto function in addition to a hydrophilic group on C4 of ring A) are essential for 5-LOX inhibitory activity.

Keywords: Leukotriene synthesis inhibitor; 5-lipoxygenase; boswellic acid; amyrin; pentacyclic triterpene; inflammation; anti-inflammatory

Introduction

5-Lipoxygenase (EC 1.13.11.34), the key enzyme of leukotriene biosynthesis, is a target for inhibitors which are thought of as potential drugs to cope with a variety of inflammation and hypersensitivity-based human diseases including asthma, arthritis, bowel diseases such as ulcerative colitis and Crohn's disease, and circulatory disorders such as shock and myocardial ischaemia (for review see: Wasserman *et al.*, 1991). Thus, for over a decade, major effort has been invested by many groups in identifying 5-lipoxygenase inhibitors, and the list of *in vitro* active 5-lipoxygenase inhibitors is vast (for reviews see: Ford-Hutchinson, 1991; Batt, 1992; McMillan & Walker, 1992; Ford-Hutchinson *et al.*, 1994). Since the 5-lipoxygenase activity is sensitive to general antioxidants, redox cyclers and radical scavengers almost all of the first generation 5-lipoxygenase inhibitors belong to the *redox-type* inhibitors. However, redox-based inhibitors are non-selective and, probably therefore, exert a variety of side-effects if applied *in vivo*.

Frankincense, the gum resin of *Boswellia serrata* and *B. carterii* has been used for the treatment of inflammatory diseases in the traditional medicine in many countries. From ethanolic extracts, which inhibited leukotriene biosynthesis in intact cells (Ammon *et al.*, 1991), boswellic acids (BAs), which belong to the ursane type pentacyclic triterpene saponines, have been identified as active principles (Safayhi *et al.*, 1992). *In vivo*, BAs prevented endotoxin and galactosamine-induced hepatitis in an animal model (Safayhi *et al.*, 1991) in which the formation of 5-lipoxygenase products from arachidonic acid is likely to be crucial for liver damage (Hagmann & Keppler, 1982: Hagmann *et al.*, 1985; Wendel & Tiegs, 1986). *In vitro*, BAs selectively blocked the leukotriene generation but did not modify either the product formation by other dioxygenases (i.e. cyclo-oxygenase-1 and 12-lipoxygenase activities in intact platelets) or the iron and ascorbate-induced peroxidation of arachidonic acid (Safayhi *et al.*, 1992). Recently we have shown that a derivative of BAs, i.e. acetyl-11-keto-β-BA (AKBA), inhibits the 5-lipoxygenase by an enzyme directed, non-competitive mechanism via binding to a pentacyclic triterpene-selective effector site (Safayhi *et al.*, 1995).

The following is an account of the structure requirements of boswellic acid type 5-lipoxygenase inhibitors for selective binding to the effector site and for enzyme inhibitory activity.

Methods

Chemicals

Amyrin (a mixture of isomeric α- and β-forms) was purchased from Roth (Karlsruhe, Germany) and olibanum in granis from Caesar & Loretz (Hilden, Germany). Acetyl-boswellic acids (Ac-BAs), as a mixture of acetylated derivatives of mainly β-BA (*c* in Table 1) and 11-keto-β-BA (*b*), were isolated from the resin of *Boswellia carterii* (olibanum) by extraction into ether, precipitation with barium hydroxide, acetylation to mixed anhydrides with acetic anhydride, cleavage of the mixed anhydrides and crystallization of the Ac-BAs from methanol

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(Winterstein & Stein, 1932). BAs were obtained by the saponification of Ac-BAs. β-BA (*c*), 11-keto-β-BA (*b*) and the corresponding acetylated derivatives were isolated by C18-rp-h.p.l.c. and characterized by infrared (i.r.), ^{1}H-n.m.r., mass (M.S.), and ultraviolet (u.v.) spectroscopy, thin layer chromatography (t.l.c.) and by their melting points as described in detail elsewhere (Safayhi *et al.*, 1992). 3α, 24-Dihydroxy-urs-12-en (3α, 24-diol), 3α,24-dihydroxy-11-oxo-urs-12-en (11-keto-diol), 3α-11-oxo-urs-12-en-24-oic acid methyl ester (11-keto-β-BA methyl ester) and 3β-acetoxy-11-oxo-urs-12-en (acetyl-11-keto-amyrin) were synthesized from β-BA and amyrin, respectively. The products were characterized by i.r., ^{1}H-n.m.r., m.s. and u.v. spectroscopy, mass and elemental analyses, t.l.c. and by their melting points. Chemical synthesis and data of analyses for the novel pentacyclic triterpene analogues will be published elsewhere.

Rat peritoneal PMNL and subcellular fractionation

Glycogen-elicited rat peritoneal PMNL were collected 4 h after the injection of a glycogen solution (5%) in Dulbecco's phosphate-buffered saline (PBS) into the peritoneum of 300–350 *g* Wistar rats as described previously (Safayhi *et al.*, 1985). The washed cells (>90% PMNL) were lysed at 2×10^7 cells ml^{-1} by sonication (3 × 5 s, Branson Sonifier) in PBS containing 1 mM EDTA, and were subjected to sequential centrifugation at 10,000 and 105,000 *g* for 10 and 60 min (4°C), respectively. Freshly obtained PMNL suspensions and aliquots of the 105,000 *g* supernatants were assayed for 5-lipoxygenase activity.

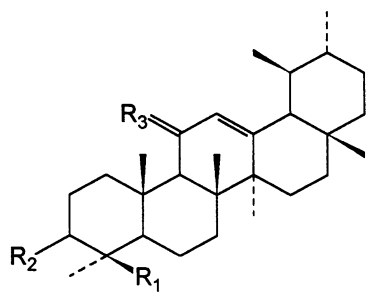
5-lipoxygenase assay

For 5-lipoxygenase product formation from endogenous arachidonic acid, glycogen-elicited rat peritoneal PMNL (1×10^7 cells) were stimulated at 37°C for 5 min by ionophore A23187 and Ca^{2+}. The 5-lipoxygenase activity in the cell-free system was tested in aliquots (corresponding to 1×10^7 cells) of the 105,000 *g* supernatant fraction of rat PMNL in the presence of 1 mM ATP, 1.8 mM $CaCl_2$, 1 mM EDTA, and 20 μM arachidonic acid at 37°C for 5 min. In intact cells and cell-free tests the incubations were terminated by cooling to 4°C and acidification to pH 3 with formic acid. For inhibition studies the test compounds dissolved in dimethyl sulphoxide (DMSO) were added 5 min prior to initiation. Inhibitors and non-inhibitory pentacyclic triterpenes were added simultaneously. All incubations including controls were carried out in the presence of 0.5% DMSO. The 5-lipoxygenase products were quantified after the addition of 170 ng PGB_2 as internal standard by extraction, rp-h.p.l.c. separation and ultraviolet detection of products as described in detail previously (Safayhi *et al.*, 1985; Ammon *et al.*, 1991). With intact rat PMNL and the 105,000 *g* supernatant fraction the main products from arachidonic acid of 5 min incubations were leukotriene B_4 (LTB_4), 6-*trans*-LTB_4, 12-epi-6-*trans*-LTB_4 and 5-HETE along with negligible amounts of 5(S), 12(S)-diHETE (Ammon *et al.*, 1991). The formation of ω-oxidized metabolites from LTB_4 was observed by intact cells only if the incubations were carried out for longer than 5 min (Safayhi *et al.*, 1985).

Measurement of 5-lipoxygenase product formation

Separation of products was performed by isocratic elution from a Shandon Hypersil C18-column (250 × 4 mm; 5 μm) with methanol/water/acetic acid = 72:28:0.2 (v/v), pH 4.8; flow rate = 1.2 ml min^{-1}. The system separates 20-OH-LTB_4, LTB_4, 6-*trans*-LTB_4, 12-epi-6-*trans*-LTB_4, (5S, 12S)-diHETE, 15-, 12- and 5-HETE, but not 5(S)-HETE and 5(S)-HPETE (retention time = 35 min at 20°C). Detection wavelength was set to 280 nm in order to detect PGB_2 (internal standard) and diHETEs or to 235 nm for H(P)ETEs. The sum of 6-*trans*-LTB_4, 12-epi-6-*trans*-LTB_4, LTB_4 and 5(S)-HETE was used for calculation of 5-lipoxygenase activity.

Table 1 Chemical structures of AKBA and analogues and their effects on 5-LOX product formation (i.e. sum of 5, 12-diHETES plus 5-HETE) from endogenous arachidonic acid in ionophore-stimulated intact rat PMNL (system A) and from 20 μM exogenous substrate in 105.000 *g* supernatants of rat PMNL (system B)



Compound	R_1	R_2	R_3	IC_{50} (μM) *system A*	IC_{50} (μM) *system B*
AKBA (a)	COOH	α-OAc	O	1.5 ± 0.2[a]	7.0 ± 2.2[a]
11-keto-β-BA (b)	COOH	α-OH	O	2.8 ± 0.2[a]	14.6 ± 7.6[a]
β-BA (c)	COOH	α-OH	2H	Partial inhibition	Partial inhibition
11-keto-diol (d)	CH_2OH	α-OH	O	4.5 ± 1.2[a]	45.3 ± 11[a]
3α,24-diol (e)	CH_2OH	α-OH	2H	No effect[b]	No effect[a]
11-keto-β-BA methyl ester (f)	$COOCH_3$	α-OH	O	No effect[b]	No effect[b]
Amyrin (g)	CH_3	β-OH	2H	No effect[b]	No effect[b]
Acetyl-11-keto-amyrin (h)	CH_3	β-OAc	O	No effect[c]	No effect[c]

[a]IC_{50}s of biological effects were determined after logit-log transformation of data of each experiment. Data of independent observations ($n = 3$) are shown as means ± s.d.
[b]No inhibition was observed in concentrations up to 50 μM.
[c]No inhibition was observed in concentrations up to 25 μM.

Solubility and critical micelle formation concentration

For each compound, quantitative solubility in the highest concentration used and the absence of micelle formation (studied by ring method with a processor tensiometer K12 V4.01, Kruess GmbH, Hamburg) was checked (data not shown).

Data

Percentage inhibition was computed by comparing values in drug groups to the value of DMSO controls. Data of independent observations (n = number of individual experiments) are shown as means ± s.d. IC_{50}s of biological effects were determined after logit-log transformation of data from each experiment.

Results and discussion

5-lipoxygenase inhibitory activity of pentacyclic triterpenes

Acetyl-11-keto-β-boswellic acid (AKBA, *a* in Table 1) inhibited the 5-lipoxygenase activity in intact cells and in the cell-free system (IC_{50} = 1.5 μM and 7 μM, respectively). Saponification of the acetyl-group of AKBA yielded 11-keto-β-BA (*b*, in Table 1) with a free 3α-OH function (Table 1 and Figure 1). This deacetylation slightly diminished the 5-lipoxygenase inhibitory potency in intact cells (IC_{50} = 3 μM) and in the cell-free system (IC_{50} = 15 μM). A further minor decrease in 5-lipoxygenase inhibitory activity was observed by reduction of the carboxyl function of 11-keto-β-BA to a primary alcohol function. The 11-keto-diol (*d*) generated still inhibited 5-lipoxygenase activity in a comparable concentration-range (IC_{50} = 4.5 μM and 45 μM in intact cells and in the cell-free system, respectively).

In order to study whether the 11-keto function of pentacyclic triterpenes is sufficient for inhibitory activity, we synthesized, starting from amyrin (*g*), acetyl-11-keto-amyrin (*h*), which in contrast to 11-keto-β-BA do not possess a carboxyl group on C-4. The novel synthetic compound, like amyrin itself, exerted no 5-lipoxygenase inhibitory activity up to 25 μM in either test system. Among the 11-keto-compounds no inhibition was also observed with the methyl ester of 11-keto-β-BA (*f*).

The β-BA (*c*), which lacks the keto-function on C-11, exerted only an incomplete, partial inhibition of the 5-lipoxygenase activity at about 60% in intact cells as well as in the cell-free system (Figure 2). The reduction of the carboxyl function of β-BA to an alcohol, yielding 3α,24-diol (*e*), caused a total loss of inhibitory activity in either system.

The data, summarized in Table 1, show that only compounds containing the 11-keto group (e.g., AKBA (*a*), 11-keto-β-BA (*b*) and 11-keto-Diol (*d*)) are able to inhibit the 5-lipoxygenase activity completely. However, a synthetic pentacyclic triterpene with a 11-keto group without a hydrophilic function on C-4 of ring A (i.e., acetyl-11-keto-amyrin (*h*)) is non-inhibitory indicating that this functional group alone is not sufficient for 5-lipoxygenase inhibitory activity. Only the combination of the 11-keto function with an additional hydrophilic group restores the inhibitory property as demonstrated with the action of AKBA, 11-keto-β-BA and 11-keto-Diol.

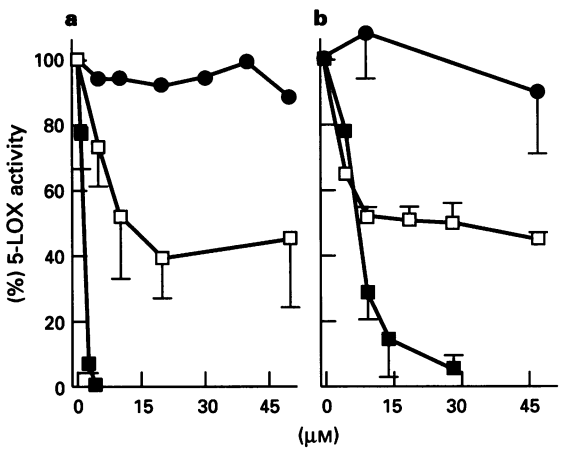


Figure 2 Effects of AKBA (■), β-BA (□) and 3α,24-diol (●) on 5-lipoxygenase (5-LOX) product formation from endogenous arachidonic acid by ionophore-stimulated intact PMNL (a) and from 20 μM exogenous substrate with 105,000 *g* supernatants of PMNL (b). Data in means (±s.d.) as percentage of 5-LOX product formation (i.e., diHETEs plus 5-HETE) in controls; n = 2–3.

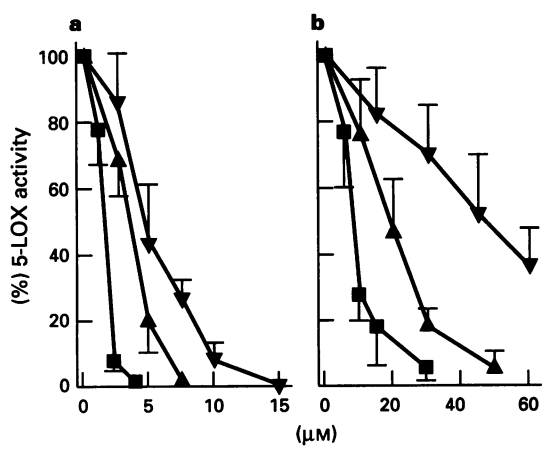


Figure 1 Effects of AKBA (■), 11-keto-β-BA (▲) and 11-keto-diol (▼) on 5-lipoxygenase (5-LOX) product formation from endogenous arachidonic acid by ionophore-stimulated intact PMNL (a) and from 20 μM exogenous substrate with 105,000 *g* supernatants of PMNL (b). Data in means (±s.d.) as percentage of 5-LOX product formation (i.e., diHETEs plus 5-HETE) in controls; n = 3.

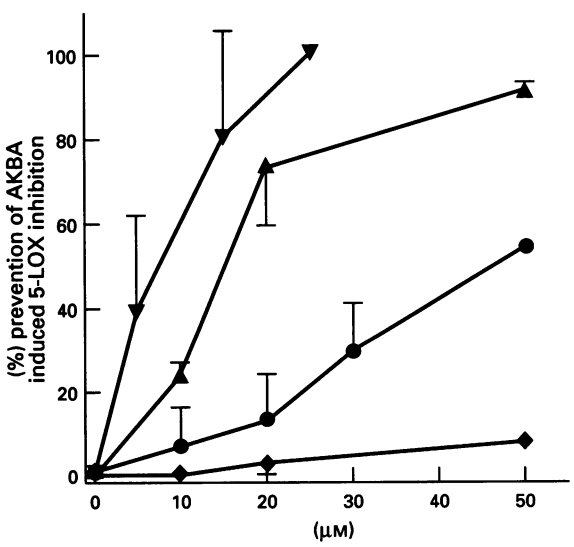


Figure 3 Prevention of 3 μM AKBA-induced 5-lipoxygenase (5-LOX) inhibition in ionophore-stimulated intact PMNL by increasing concentrations of non-inhibitory pentacyclic triterpenes: acetyl-11-keto-amyrin (▼), amyrin (▲), 3α,24-diol (●) and 11-keto-β-BA methyl ester (◆). Data in means ±s.d. as percentage of 5-LOX product formation (i.e., diHETEs plus 5-HETE) in controls; n = 3.

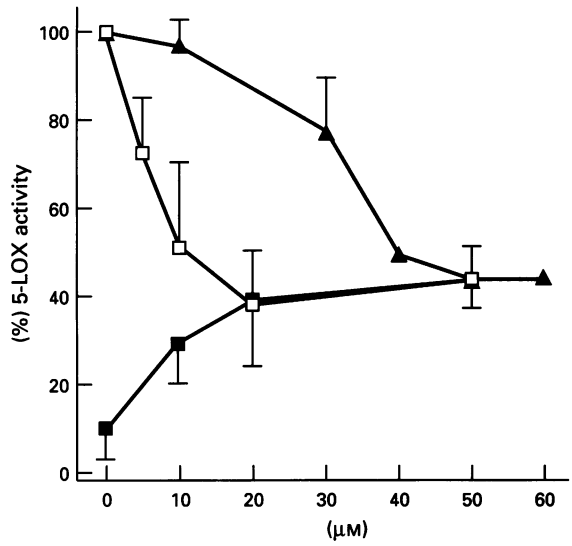


Figure 4 Partial inhibition of 5-lipoxygenase (5-LOX) activity in ionophore-stimulated intact PMNL by β-BA (□) and by β-BA in the presence of 3 μM AKBA (■) or 30 μM amyrin (▲). Data in means ± s.d. as percentage of 5-LOX product formation (i.e. diHETEs plus 5-HETE) in controls; $n = 3$.

Effector site binding of pentacyclic triterpenes

In order to distinguish variations in binding affinity from modification of the intrinsic inhibitory activity we performed studies on functional antagonism of inhibitory, partial inhibitory and non-inhibitory analogues. As shown in Figure 3 the presence of increasing concentrations of the non-inhibitory compound acetyl-11-keto-amyrin (*h*), like amyrin (*g*) itself, prevented the 3 μM AKBA induced 5-lipoxygenase inhibition in intact cells. The 5-lipoxygenase inhibition by AKBA was also antagonized by 3α,24-diol (*e*), however, not by 11-keto-β-BA methyl ester (*f*), indicating that methylation leads to a drastic decrease in binding affinity.

In order to confirm a unique binding site for complete and partial inhibitors we investigated whether partially inhibitory compounds of this class can at least partially antagonize the inhibitory effect of AKBA on 5-lipoxygenase activity. Figure 4 shows that increasing concentrations of the incomplete-inhibitor β-BA (*c*) partially prevented the enzyme inhibition by AKBA. The partial antagonistic effect led to a resulting 5-lipoxygenase product synthesis of about 40% (vs. non-inhibited controls), corresponding to the inhibitory effect of 50 μM β-BA. The presence of the non-inhibitory pentacyclic triterpene amyrin in a concentration of 30 μM shifted the concentration-inhibition curve of β-BA to the right, indicating that the incomplete effect of β-BA is not due to solubility limitations (Figure 4).

The present data reveal that we have to distinguish between binding site affinity and 5-lipoxygenase inhibitory activity of pentacyclic triterpenes. In summary, the data suggest that the binding of pentacyclic triterpenes to the effector site is mediated by the pentacyclic ring system whereas defined functional groups, especially the 11-keto function, are required for 5-lipoxygenase inhibitory activity. Modifications of functional groups of AKBA lead to 5-lipoxygenase inhibitory, partial-inhibitory and non-inhibitory ligands of this highly pentacyclic triterpene selective binding site, or to an analogue (i.e. the methyl ester of 11-keto-β-BA) with a decreased affinity. With the exception of the latter 11-keto-β-BA methyl ester (*f*) all non-inhibitory pentacyclic triterpenes antagonize the AKBA-induced 5-lipoxygenase inhibition most probably due to a competition for a common binding site. Since AKBA inhibits not only the 5-lipoxygenase in intact cells but also the affinity chromatography purified 5-lipoxygenase action in a minimal system (Safayhi *et al.*, 1995) it is likely that the not yet characterized novel drug binding site for pentacyclic triterpenes is located on the enzyme.

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