0026-895X/95/061212-05$3.00/0

MOLECULAR PHARMACOLOGY, 47:1212–1216 (1995).

Mechanism of 5-Lipoxygenase Inhibition by Acetyl-11-keto-β-boswellic Acid

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Received November 21, 1994; Accepted March 24, 1995

SUMMARY

The formation of 5-lipoxygenase (EC 1.13.11.34) products from endogenous substrate by intact rat neutrophilic granulocytes and from exogenous arachidonic acid by rat granulocyte 105,000 × *g* supernatants and affinity chromatography-purified human leukocyte 5-lipoxygenase was inhibited by acetyl-11-keto-β-boswellic acid (IC_{50} values of 1.5 μM, 8 μM, and 16 μM, respectively). With other pentacyclic triterpenes lacking the 11-keto function and/or the carboxyl function on ring A (e.g., amyrin and ursolic acid), no 5-lipoxygenase inhibition was observed. The presence of the noninhibitory pentacyclic triterpenes both in intact cells and in the cell-free system caused a concentration-dependent reversal of the 5-lipoxygenase inhibition by acetyl-11-keto-β-boswellic acid, whereas the inhibitory actions of 5-lipoxygenase inhibitors from different chemical classes (MK-886, L-739,010, ZM-230,487, and nordihydroguaiaretic acid) were not modified. The inhibition by acetyl-11-keto-β-boswellic acid and the antagonism by noninhibitory pentacyclic triterpenes were not due to nonspecific lipophilic interactions, because lipophilic four-ring compounds (cholesterol, cortisone, and testosterone) neither inhibited the activity of the 5-lipoxygenase nor antagonized the inhibitory action of acetyl-11-keto-β-boswellic acid. Therefore, we conclude that acetyl-11-keto-β-boswellic acid acts directly on the 5-lipoxygenase enzyme at a selective site for pentacyclic triterpenes that is different from the arachidonate substrate binding site.

Owing to diverse and potent proinflammatory properties, leukotrienes have been implicated as putative mediators in inflammation and hypersensitivity (1–3). The development of clinically useful inhibitors of 5-lipoxygenase, the key enzyme of leukotriene synthesis, has become the subject of intensive research (4–6).

From the gum resin of *Boswellia serrata*, which has been used as an antiphlogistic remedy in traditional medicine in India and other eastern countries, Ac-BAs were identified as biologically active components; Ac-BAs have been shown to inhibit leukotriene synthesis from endogenous arachidonic acid in intact PMNL by a non-redox-based mechanism and, in contrast to many nonselective, redox-type inhibitors, not to modify 12-lipoxygenase and cyclooxygenase-1 activities in platelets (7).

Here we report on the 5-lipoxygenase-directed leukotriene synthesis inhibition by AKBA, by a novel mechanism. We present data on the selective functional antagonism of pentacyclic triterpenes with and without inhibitory activity, suggesting a specific binding site that is different from the substrate binding region.

The investigation was supported by the Deutsche Forschungsgemeinschaft (Grant Sa.561/2–1).

Materials and Methods

Chemicals. LTB_4, 20-OH-LTB_4, 6-*trans*-LTB_4, 12-epi-6-*trans*-LTB_4, 5-H(P)ETE, 12-HETE and 15-HETE as RP-HPLC standards, PGB_2 as intrinsic standard, ionophore A23187, NDGA, testosterone, cortisone, cholesterol, catalase, glycogen (type IX from bovine liver), soybean trypsin inhibitor, L-α-phosphatidylcholine (type III from egg yolk), ATP (disodium salt), ATP-agarose (ATP immobilized with 9-atom spacers on 4% beaded agarose), dithiothreitol, and arachidonic acid (sodium salt) were obtained from Sigma (Deisenhofen, Germany). Phenylmethylsulfonyl fluoride was purchased from Serva (Heidelberg, Germany). Amyrin (a mixture of α- and β-isomers) and ursolic acid (Rotichrom) (both gas chromatography grade) were purchased from Roth (Karlsruhe, Germany). Ac-BAs were isolated from

ABBREVIATIONS: Ac-BA, acetylboswellic acid; AKBA, acetyl-11-keto-β-boswellic acid; A-64077, *N*-(1-benzo[*b*]thien-2-ylethyl)-*N*-hyroxyurea; AA-861, 2,3,5-trimethyl-6-(12-hydroxy-5–10-dodecadiynyl)-1,4-benzoquinone; BAY X1005, (*R*)-2-[4-(quinolin-2-ylmethoxy)phenyl]-2-cyclopentyl acetic acid; HETE, hydroxy-6,8,11,14-eicosatetraenoic acid; HPETE, hydroperoxy-6,8,11,14-eicosatetraenoic acid; 5,12-diHETE, 5,12-dihydroxy-6,8,10,14-eisosatetraenoic acid; FLAP, 5-lipoxygenase-activating protein; L-739,010, (1*S*,5*R*)-3-cyano-1-(3-furyl)-6-[6-[3-(3α-hydroxy-6,8-dioxabicyclo[3,2,1]octanyl)]pyridin-2-ylmethoxy]naphthalene; LTB_4, leukotriene B_4 [(5*S*,12*R*)-dihydroxy-6,8,10,14-eicosatetraenoic acid]; MK-886, 3-[1-(4-chlorobenzyl)-3-*t*-butylthio-*t*-isopropylindol-2-yl]-2,2-dimethylpropanoic acid; NDGA, nordihydroguaiaretic acid; PBS, Dulbecco's phosphate-buffered saline; PGB_2, prostaglandin B_2; PMNL, polymorphonuclear leukocytes; ZM-230,487, 6-[[3-fluoro-5-(4-methoxy-3,4,5,6-tetrahydro-2*H*-pyran-4-yl)phenoxy]methyl]-1-ethylquinol-2-one; RP, reverse phase; DMSO, dimethylsulfoxide; HPLC, high performance liquid chromatography.

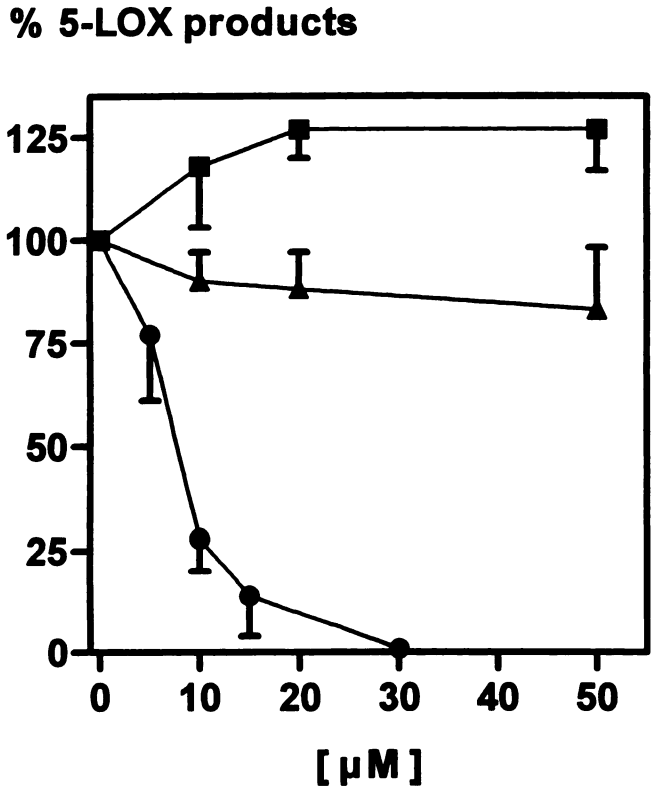

Fig. 1. Effects of AKBA (●), amyrin (▲), and ursolic acid (■) on 5-lipoxygenase (*5-LOX*) product formation from 20 μM exogenous arachidonic acid by Ca^{2+}- and 1 mM ATP-stimulated 105,000 × *g* supernatants of rat peritoneal PMNL. The 100% value is the sum of 330 ± 90 pmol of LTB_4, 295 ± 60 pmol of 6-*trans*-LTB_4 and 12-epi-6-*trans*-LTB_4 isomers, and 1290 ± 280 pmol of 5-HETE. Data are means ± standard errors of percentage of product synthesis in controls (n = 3).

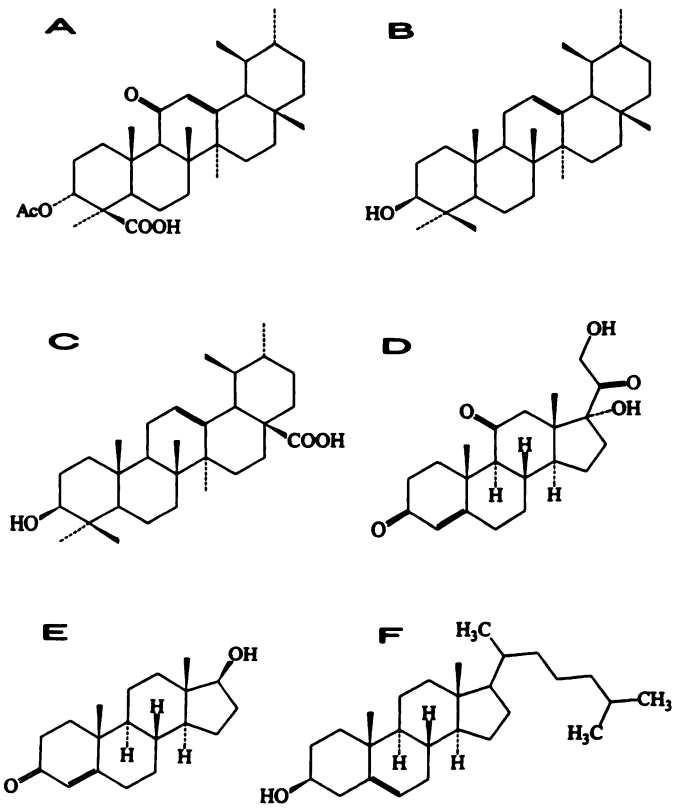

Fig. 2. Chemical structures of AKBA (A), α-amyrin (B), ursolic acid (C), cortisone (D), testosterone (E), and cholesterol (F).

the gum resin of *B. serrata* or *Boswellia carteri* by extraction of the boswellic acids into ether, precipitation with barium hydroxide, acetylation to mixed anhydrides with acetic acid, cleavage of the mixed anhydrides, and crystallization of the Ac-BAs from methanol (8).

AKBA was separated from other Ac-BAs by C_{18} RP-HPLC and was characterized by IR, ^1H NMR, mass, and UV spectroscopy, thin layer chromatography, and melting point determination (7). MK-886 (formerly designated L-663,536) and L-739,010 were kind gifts from Dr. A. W. Ford-Hutchinson (Merck Frosst Centre for Therapeutic Research, Kirkland, Canada). ZM-230,487 (formerly ICI-230,487; the *N*-ethyl analogue of ICI-D2138) was a kind gift from Dr. G. C. Crawley (ICI-Zeneca Pharmaceuticals, Macclesfield, Cheshire, England).

Rat peritoneal PMNL fraction and subcellular fractionation. Glycogen-elicited rat peritoneal PMNL were collected 4 hr after the injection of a glycogen solution (5%, in PBS) into the peritoneum of 300–350-g Wistar rats, as described previously (9). The washed cells (>90% PMNL) were lysed at 2 × 10^7 cells/ml by sonication (3 × 5 sec; 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 (at 4°), respectively. Freshly obtained PMNL suspensions and aliquots of the 105,000 × *g* supernatants were assayed for 5-lipoxygenase activity.

Human leukocytes and subcellular fractionation. Human blood leukocytes were prepared from fresh buffy coat fractions (obtained from Transfusionsmedizinische Abteilung, Universitaetsklinikum, Tuebingen, Germany) by density gradient centrifugation with Polymorphprep (Nycomed) and hypotonic lysis of contaminating erythrocytes by a 30-sec incubation of the cells in a solution of 155 mM NH_4Cl, 0.1 mM EDTA, and 10 mM $KHCO_3$. For subcellular fractionation, leukocytes were lysed at 1.2 × 10^8 cells/ml of PBS (supplemented with 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20 μg/ml catalase, and 60 μg/ml soybean trypsin inhibitor) by sonication (3 × 5 sec) and were subjected to sequential centrifugation at 10,000 and 105,000 × *g* for 10 and 60 min (at 4°), respectively.

5-Lipoxygenase purification. 5-Lipoxygenase was purified from the 105,000 × *g* supernatant fraction of human blood leukocytes by ATP-agarose affinity chromatography, with minor modifications of the protocol for aerobic conditions as described previously (10, 11). With the modifications, the 105,000 × *g* supernatant fraction was loaded onto a 2-ml ATP-agarose affinity column in PBS supplemented as described for the subcellular fractionation of human leukocytes, and the column was rotated for 30 min at 4° for maximal binding. This protocol yielded about 500 μg of protein from 8 × 10^8 cells. Enzyme was not stored but was immediately tested 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° for 5 min with 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° for 5 min. For assay of affinity column-purified 5-lipoxygenase, the enzyme reaction was performed in PBS containing 1 mM EDTA, 1.8 mM $CaCl_2$, 2 mM ATP, 20 μM arachidonic acid, 20 μg/ml phosphatidylcholine, 0.5 mM dithiothreitol, and 25 μg/ml γ-globulin, in a final volume of 1 ml. Stimulatory leukocyte protein fractions were not added to the incubations (for review, see Ref. 12). The reaction was initiated by enzyme addition (corresponding to enzyme from 4 × 10^7 cells) and was carried out for 10 min at 25°.

In all test systems for 5-lipoxygenase activity (intact cells, cell-free

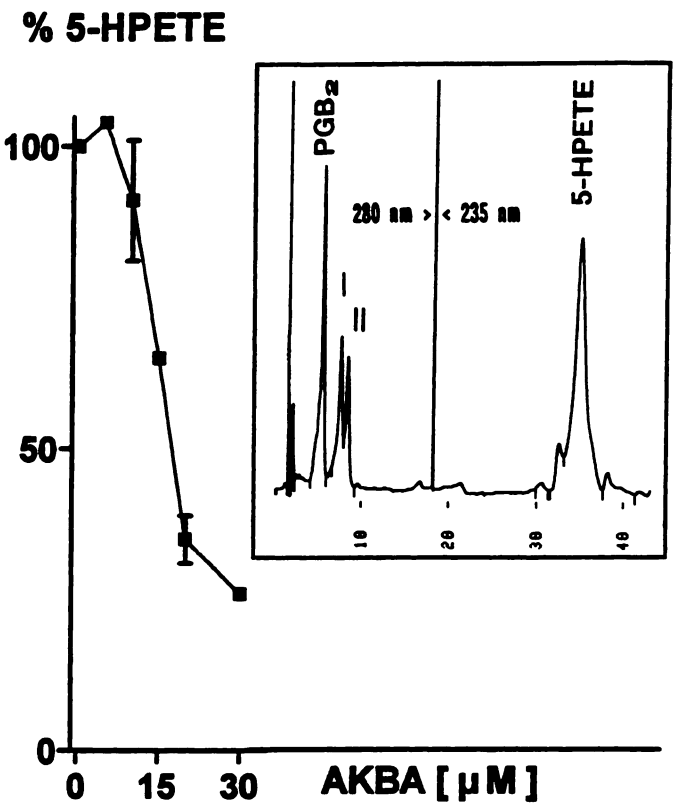

Fig. 3. Inhibition by AKBA of affinity-purified 5-lipoxygenase from human granulocytes. ATP-agarose affinity chromatography-purified human leukocyte 5-lipoxygenase was stimulated at 25° for 10 min in the presence of 1 mM EDTA, 1.8 mM $CaCl_2$, 2 mM ATP, 20 μM arachidonic acid, 20 μg/ml phosphatidylcholine, 0.5 mM dithiothreitol, and 25 μg/ml γ-globulin. Data are means or means ± standard deviations of experiments (n = 2 or 3) with enzyme from different purifications, presented as percentage of 5-HPETE synthesis in controls (100% = 75 nmol of 5-HPETE/mg of protein/10 min). *Inset*, HPLC elution profile of affinity chromatography-purified human leukocyte 5-lipoxygenase products from exogenous substrate in a representative control incubation with vehicle (0.5% DMSO). PGB_2 was the internal standard. *I*, 5(*S*),12(*R*)-6-*trans*-LTB_4; *II*, 5(*S*),12(*S*)-6-*trans*-LTB_4.

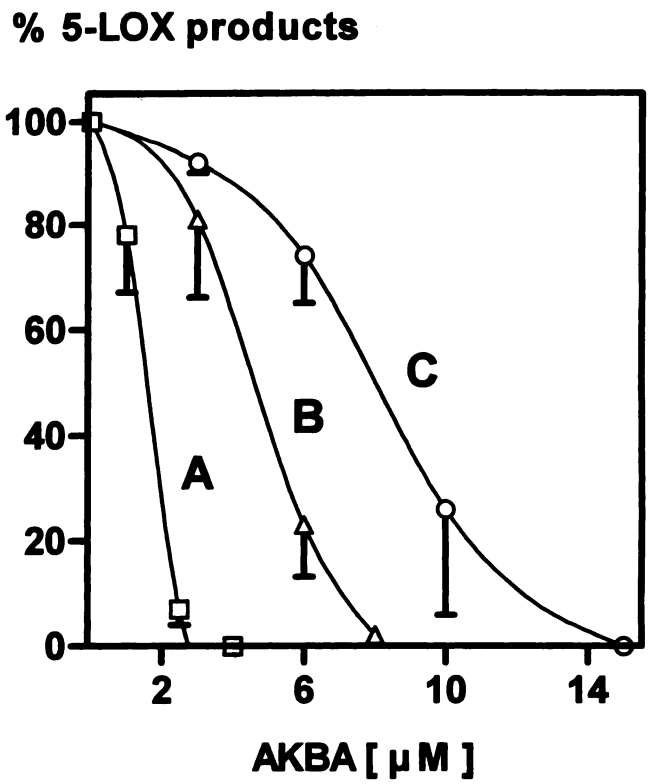

Fig. 4. Inhibition by AKBA of 5-lipoxygenase (*5-LOX*) product formation from endogenous arachidonic acid in Ca^{2+}- and ionophore-stimulated rat peritoneal PMNL in the absence (*curve A*) and presence of amyrin (*curve B*, 30 μM; *curve C*, 50 μM). Data are means ± standard deviations of percentage of product synthesis in controls (n = 3).

system, and purified enzyme system), the incubations were terminated by cooling to 4° and acidification to pH 3 with formic acid. For inhibition studies, the test compounds (dissolved in DMSO) were added before initiation. 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 of PGB_2 (as internal standard), by extraction, RP-HPLC separation, and UV detection of products, as described in detail previously (7, 9).

With intact rat PMNL, human leukocytes, and the 105,000 × g supernatant fraction of rat PMNL, the main arachidonic acid products from 5-min incubations were LTB_4, 6-*trans*-LTB_4, 12-epi-6-*trans*-LTB_4, and 5-HETE, as well as negligible amounts of 5(*S*),12(*S*)-diHETE (13). The formation of ω-oxidized metabolites from LTB_4 was observed with intact cells only if the incubations were carried out for >5 min (9). In incubations with purified enzyme, the main product from 20 μM arachidonic acid was 5-HPETE (75 nmol/mg of protein/10 min), with minor quantities of 6-*trans*- and 12-epi-6-*trans*-LTB_4 isomers (7 and 5 nmol/mg of protein/10 min, respectively).

Measurement of 5-lipoxygenase product formation. Separation of products was performed by isocratic elution from a Shandon C_{18} 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). The system separates 20-OH-LTB_4, LTB_4, 6-*trans*-LTB_4, 12-epi-6-*trans*-LTB_4, 5(*S*),12(*S*)-diHETE, 15-HETE, 12-HETE, and 5-HETE, but not 5(*S*)-HETE and 5(*S*)-HPETE (retention time, 35 min at 20°) (13). Detection wavelength was set to 280 nm for PGB_2 (internal standard) and diHETEs or to 235 nm for H(P)ETEs.

Protein measurement. Protein was quantified according to the method of Bradford (14).

Data analysis. Percentage inhibition was computed by comparing values for treatment groups with the values for individual controls. Data from independent observations (n = number of observations) are shown as means ± standard deviations.

Results

Among the boswellic acids, AKBA was previously shown to be the most potent inhibitor (IC_{50} = 1.5 μM) of ionophore- and calcium-elicited leukotriene synthesis from endogenous arachidonic acid in intact PMNL (7). As demonstrated in Fig. 1, with 105,000 × g supernatants of rat peritoneal PMNL AKBA inhibited the 5-lipoxygenase product formation from 20 μM exogenous substrate (in the presence of 1.8 μM Ca^{2+}, 1 mM EDTA, and 1 mM ATP) concentration-dependently, with comparable efficiency (IC_{50} = 8 μM). In the cell-free system the 5-lipoxygenase product formation depended on exogenous substrate availability (data not shown) but the IC_{50} values for AKBA did not, e.g., identical IC_{50} values of 8 μM were observed in the range of 5–20 μM arachidonic acid. In contrast to AKBA, other natural pentacyclic triterpenes (amyrin and ursolic acid) did not inhibit 5-lipoxygenase activity in the cell-free system at comparable concentrations (Fig. 1; structures in Fig. 2). In a minimal incubation mixture (containing Ca^{2+}, ATP, phosphatidylcholine, γ-globulin, and arachidonic

acid only), AKBA inhibited 5-HPETE formation by affinity-purified 5-lipoxygenase with an IC_{50} value of 16 μM (Fig. 3).

Fig. 4 shows that in intact cells the presence of increasing concentrations of the noninhibitory pentacyclic triterpene amyrin shifted the concentration-inhibition curve of AKBA to the right. The IC_{50} value for AKBA was 1.5 μM in the absence of amyrin and was increased to 6 and 8 μM by 30 and 50 μM amyrin, respectively.

In an alternative system, 5-lipoxygenase product formation in ionophore/calcium-challenged intact PMNL was blocked to 10% residual activity by 5-lipoxygenase inhibitors from different chemical classes. In this system the IC_{50} value for the nonselective, redox-based lipoxygenase and cyclooxygenase inhibitor NDGA was 0.5 μM. The 5-lipoxygenase-selective, enzyme-directed, non-redox-based inhibitors L-739,010 (15) and ZM-237,487 (16) were inhibitory, with IC_{50} values of 2 nM and 5 nM, respectively, as was the so-called "translocation inhibitor" MK-886 (17), with an IC_{50} of 10 nM. The presence of increasing concentrations of amyrin caused a reversal of the inhibitory action of 3 μM AKBA. In contrast to the functional antagonism of the inhibitory and noninhibitory pentacyclic triterpenes (AKBA versus amyrin) in ionophore-challenged cells, the inhibitory effects of NDGA, L-739,010, ZM-230,487, and MK-886 were not modified by the presence of increasing concentrations of amyrin (Fig. 5). Fig. 6 demonstrates that also in 105,000 × *g* supernatants the presence of increasing concentrations of noninhibitory pentacyclic triterpenes (amyrin or ursolic acid) caused a reversal of the AKBA-induced inhibition of 5-lipoxygenase product formation from exogenous arachidonic acid.

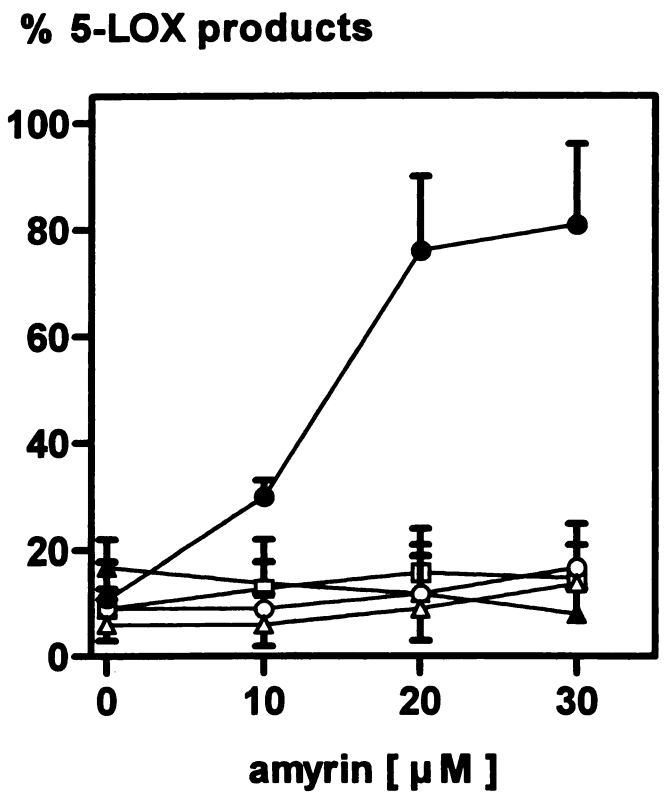

Fig. 5. Inhibition by 3 μM AKBA (●), 0.75 μM NDGA (○), 15 nM MK-886 (△), 3 nM L-739,010 (□), or 10 nM ZM-230,487 (▲) of 5-lipoxygenase (*5-LOX*) product formation in Ca^{2+}- and ionophore-stimulated intact rat peritoneal PMNL, in the absence or presence of increasing concentrations of amyrin. Data are means ± standard deviations of percentage of product synthesis in controls (n = 3).

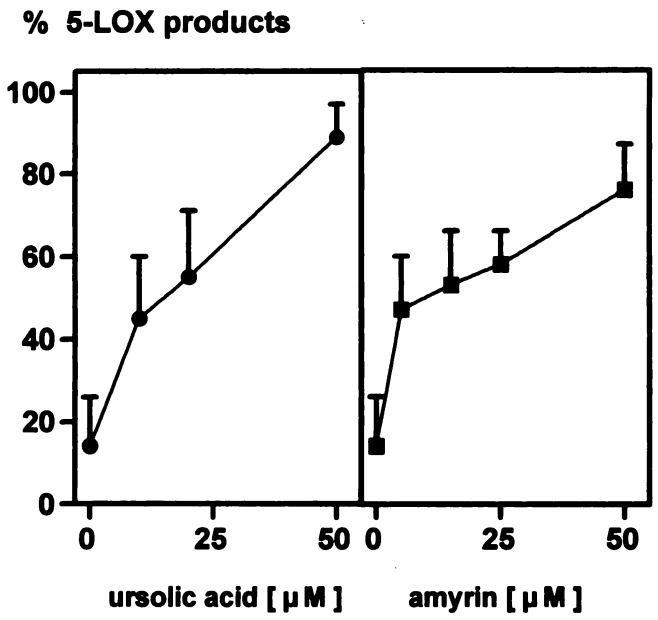

Fig. 6. Inhibition by 15 μM AKBA of 5-lipoxygenase (*5-LOX*) product formation from exogenous arachidonic acid (20 μM) in 105,000 × *g* supernatants of rat peritoneal PMNL, in the absence or presence of increasing concentrations of amyrin (*right*) or ursolic acid (*left*). Data are means ± standard deviations of percentage of product synthesis in controls (n = 3).

To study whether the antagonistic effects of inhibitory and noninhibitory pentacyclic triterpenes are due to nonselective lipophilic interactions, the effects of lipophilic compounds with a planar four-ring system (e.g., cholesterol, cortisone, and testosterone) were tested in the intact cell system. As shown in Table 1, the four-ring compounds, at comparable concentrations, did not modify the 5-lipoxygenase activity or reverse the AKBA-induced inhibition of 5-lipoxygenase product formation.

TABLE 1

5-Lipoxygenase product formation from endogenous arachidonic acid in Ca^{2+}- and ionophore-elicited PMNL and its inhibition by 3 μM AKBA, in the absence or presence of lipophilic, planar, four-ring compounds (50 μM cortisol, 50 μM testosterone, and 25 μM cholesterol) or 30 μM amyrin

Data are means ± standard deviations (n = 3).

AKBA	Compound	5-Lipoxygenase products		
		LTB_4	LTB_4 isomers[a]	5-HETE
μM	μM	*pmol of product/10^6 cells*		
0	None	41 ± 10	26 ± 6	77 ± 31
0	Testosterone, 50	39 ± 8	21 ± 5	89 ± 40
0	Cortisol, 50	38 ± 8	24 ± 7	72 ± 18
0	Cholesterol, 25	36 ± 3	21 ± 4	74 ± 16
0	Amyrin, 30	43 ± 13	15 ± 5	75 ± 39
3	None	3 ± 3	0	0
3	Testosterone, 50	3 ± 3	0	0
3	Cortisol, 50	4 ± 4	0	0
3	Cholesterol, 25	3 ± 3	0	2 ± 3
3	Amyrin, 30	32 ± 5	11 ± 3	50 ± 9

[a] LTB_4 isomers, 6-*trans*-LTB_4 plus 12-epi-6-*trans*-LTB_4.

Discussion

The inhibition by AKBA of 5-lipoxygenase product synthesis from exogenous arachidonic acid in the cell-free system and with purified enzyme suggests that this inhibitor acts on leukotriene biosynthesis at a point distal to the substrate release by phospholipase A_2. The observations further indicate that membrane factors (especially the presence of FLAP) are not crucial for the inhibitory action of AKBA. The sensitivity of affinity-purified 5-lipoxygenase to inhibition by AKBA suggests that the presence of postulated (but not yet identified) stimulatory leukocyte protein factors is not a prerequisite for the inhibitory effect of AKBA on 5-lipoxygenase action. Thus, the effector site for AKBA is likely to be located on the enzyme.

The inhibitory potencies of AKBA in intact cells, in the cell-free system, and with purified enzyme differ only by a factor of 4–8 (IC_{50} values of 1.5, 8 μM, and 16 μM, respectively). This is in line with the experience of others. For direct 5-lipoxygenase inhibitors [e.g., A-64077 (zileuton) and AA-861] the IC_{50} values for inhibition of leukotriene synthesis in intact cells and in the 10,000 $\times$ g supernatants were determined to differ by roughly 1 order of magnitude, whereas for the quinoline- and indole-type translocation inhibitors, which mainly impair the interaction of FLAP, arachidonic acid, and 5-lipoxygenase at the membrane site, about 1000-fold higher concentrations were required to inhibit leukotriene formation in the cell-free system, compared with intact cells (18, 19).

Both in intact cells and in the cell-free test system, the inhibitory action of AKBA was antagonized by noninhibitory pentacyclic triterpenes. In contrast to the action of amyrin and ursolic acid, the presence of noninhibitory, lipophilic, four-ring compounds caused no reversal of the inhibition by AKBA. Thus, the data indicate that the antagonistic effects of noninhibitory pentacyclic triterpenes are not due to a nonselective lipophilic interaction. Therefore, it is likely that the pentacyclic triterpene ring system is crucial for the observed inhibitory and antagonistic effects on 5-lipoxygenase.

The observation of the reversal by noninhibitory pentacyclic triterpenes of AKBA-induced 5-lipoxygenase inhibition also reveals that inhibitory pentacyclic triterpenes do not act in a competitive manner, because, in the case of a competitive mechanism for 5-lipoxygenase inhibition by AKBA, antagonism with inhibitory and noninhibitory pentacyclic triterpenes should not be possible. This interpretation is in line with the observation that the inhibitory potency of AKBA is not modified by variation of the substrate concentration in the cell-free system.

Furthermore, the antagonizing effect of noninhibitory pentacyclic triterpenes is selective for the pentacyclic triterpene-type inhibitor AKBA, because the inhibitory actions of various other 5-lipoxygenase inhibitors from different chemical classes, i.e., the nonselective, redox-based, lipoxygenase inhibitor NDGA (7), the translocation inhibitor MK-886 (17), and the non-redox-based, enzyme-directed, 5-lipoxygenase inhibitors L-739,010 (15) and ZM-230,487 (16), were not modified by the noninhibitory pentacyclic triterpene amyrin. A competitive mechanism, i.e., interaction with the binding of arachidonic acid to 5-lipoxygenase and/or FLAP, is suggested for most of the non-redox-based inhibitors, including the 5-lipoxygenase-directed inhibitors from the dioxabicyclooctanyl naphthalene and methoxytetrahydropyran series (exemplified by L-739,010 and ZM-230,487, respectively) and the FLAP-directed translocation inhibitors (exemplified by MK-886 and BAY X1005) (6, 18, 20). In summary, we conclude that AKBA is an enzyme-directed, non-redox-based, leukotriene biosynthesis inhibitor that interacts with 5-lipoxygenase via a pentacyclic triterpene-selective binding site that is different from the arachidonic acid substrate binding site.

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