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Boswellic Acids: Novel, Specific, Nonredox Inhibitors of 5-Lipoxygenase

HASAN SAFAYHI, THOMAS MACK, JOACHIM SABIERAJ, MICHAEL I. ANAZODO, LAKSHMINARAYANAPURAM R. SUBRAMANIAN[1] and HERMANN P. T. AMMON

Department of Pharmacology, Pharmaceutical Institute, University of Tuebingen, Auf der Morgenstelle 8, 7400 Tuebingen, FRG

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ABSTRACT

Isomers (α- and β-) of boswellic acids (BAs), 11-keto-β-BA and their acetyl derivatives were isolated from the gum resin of *Boswellia serrata*. BA and derivatives concentration dependently decreased the formation of leukotriene B_4 from endogenous arachidonic acid in rat peritoneal neutrophils. Among the BAs, acetyl-11-keto-β-BA induced the most pronounced inhibition of 5-lipoxygenase (5-LO) product formation with an IC_{50} of 1.5 μM. In contrast to the redox type 5-LO inhibitor nordihydroguaiaretic acid, BA in concentrations up to 400 μM did not impair the cyclooxygenase and 12-lipoxygenase in isolated human platelets and the peroxidation of arachidonic acid by Fe-ascorbate. The data strongly suggest that BAs are specific, nonreducing-type inhibitors of the 5-LO product formation either interacting directly with the 5-LO or blocking its translocation.

The gum resin of *Boswellia serrata* has been used for the treatment of inflammatory diseases in the traditional Ayurvedic medicine in India and other countries in the east. The crude extract of the gum resin, also available on market in India under the trade name "Sallaki," was shown previously to reduce carrageenan-induced paw edema in normal rats and mice, as well as in adrenalectomized rats, but exerted no analgesic or antipyretic effects (Singh and Atal, 1986). Very recently we demonstrated that the ethanolic extract of the resin decreased LTB_4 production in rat peritoneal neutrophils *in vitro* (Ammon *et al.*, 1991). In addition, we observed that BAs isolated from the gum resin prevented galactosamine-endotoxin-induced liver injury in mice (Safayhi *et al.*, 1991).

In the present communication we report on the isolation and characterization of BAs from the gum resin exudate of *Boswellia serrata* as the biologically active principles for the 5-LO inhibition. We demonstrate that BAs are highly specific for the 5-LO activity and that the underlying mechanism is not due to antioxidative properties.

Materials and Methods

Materials

12-HHT, 12-HETE, PGB_2, NDGA, indomethacin, glycogen (type IX from bovine liver), Ca^{++}-ionophore A23187 and arachidonic acid (sodium salt) were supplied by Sigma Chemical Co. (Deisenhofen, FRG). Four standard diHETE isomers as well as 5-(*S*)-HETE, 12-(*S*)-HETE and 15-(*S*)-HETE from biological origin were a kind gift of Dr. G. Loschen and Dr. W. Martin (Grünenthal, FRG). 18-β-GA was obtained from Roth (Karlsruhe, FRG). All compounds not listed were purchased from Merck (Darmstadt, FRG) in research grade quality. The prepacked rp-HPLC columns (Shandon Hypersil C_{18}, 5 μm, 250 × 4 mm and 250 × 10 mm) were supplied by Bischoff (Leonberg, FRG) and Novo Pak (C_{18}, 3 μm, 3.9 × 150 mm) by Waters (Framingham, MA).

Purification of BAs. Extraction and purification of BAs were carried out according to Winterstein and Stein (1932). From 2 kg of the gum resin of *Boswellia serrata* we obtained 285 g of the barium salt of BA. Two hundred fifty grams of the barium salt yielded 60 g of the mixed anhydride of acetic acid and BA. From 50 g of the mixed anhydride 32 g of acetyl-BA were obtained after boiling with ether-MeOH and crystallization from MeOH. Aliquots of acetyl-BA were saponified with methanolic KOH, crystallized from MeOH, washed with water and dried under vacuum at 140°C. The α- and β-isomers of BA and the 11-keto-β-BA as well as the acetyl-BA were isolated by HPLC (Shandon C_{18}, 250 × 10 mm, MeOH-water-acetic acid = 90:10:0.2, pH 3.9, 4 ml/min). From 600 mg of acetyl-BA, 475 mg of acetyl-β-BA, 30 mg of acetyl-11-keto-β-BA and 15 mg of acetyl-α-BA were regained after rp-HPLC separation; 1000 mg of BA yielded 880 mg of β-BA, 30 mg of 11-keto-β-BA and 15 mg of α-BA. The isolated BA and their 11-keto- and acetyl-derivatives were characterized by MS, IR, UV and ^{1}H-NMR spectra and by their melting points.

β-BA. In the IR spectrum, peaks due to hydroxy and OH of carboxylic group at ν = 3500 and 3400 cm^{-1}, and for carbonyl group at ν = 1700 cm^{-1} were observed. The ^{1}H-NMR spectrum showed signals for one vinylic proton at δ = 5.15 (1H, br s) and for the C-3 methine proton

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[1]Present address: Institute for Organic Chemistry, University of Tuebingen, Auf der Morgenstelle 18, 7400 Tuebingen, FRG.

ABBREVIATIONS: LT, leukotriene; BA, boswellic acid; LO, lipoxygenase; 12-HHT, 12-hydroxyheptadecatrienoic acid; HETE, hydroxyeicosatetraenoic acid; PG, prostaglandin; NDGA, nordihydroguaiaretic acid; diHETE, dihydroxyeicosatetraenoic acid; β-GA, 18-β-glycyrrhetinic acid; rp-HPLC, reversed phase high-pressure liquid chromatography; MS, mass spectrometry; IR, infrared; PMNL, polymorphonuclear leukocytes; CO, cyclooxygenase.

at δ = 4.1 (1H, br s). In the MS the typical base peak was observed at m/z = 218 due to the ion arising as a result of characteristic retro-Diels-Alder fragmentation of the ring C of Δ^{12}-oleanane-ursane derivatives without any substituent on ring D and E (Budzikiewicz *et al.*, 1963), which is characteristic for β-BA (Pardhy and Bhattacharyya 1978). The molecular ion peak was observed at m/z = 456. Melting point: 229°C.

Acetyl-β-BA. The IR spectrum showed the carbonyl of the acetoxy group at ν = 1730 cm^{-1}. In the ^{1}H-NMR the peak for C-12 vinylic proton appeared as a singlet at δ = 5.3. The molecular ion peak was observed at m/z = 498 in the MS. Saponification of acetyl-β-BA afforded β-BA. The analytical and spectral data of the product were identical with an authentic sample of β-BA. Melting point = 255°C.

Acetyl-11-keto-β-BA. The IR spectrum showed an α,β-unsaturated carbonyl function at ν = 1658 (C = O) and 1610 (C = C) besides and acetoxy group at ν = 1735 cm^{-1} (C = O of acetate). This is confirmed further by UV-absorption spectrum (λ_{max} = 250 nm). The ^{1}H-NMR data revealed the presence of one vinylic proton at δ = 5.55 (s) shifted downfield, being the proton on the double bond of a α,β-unsaturated carbonyl system. The molecular ion peak in the MS was observed at m/z = 512, and was indicative of the presence of a 11-keto-oleanane-ursane fundamental in the structure. Melting point: 279°C.

11-keto-β-BA. 11-keto-β-BA was obtained by the saponification of acetyl-11-keto-β-BA. The compound showed an α,β-unsaturated carbonyl function at ν = 1680 (C = O) and 1635 cm^{-1} (C = C) as evident from the IR spectrum. UV absorption possessed a maximum at λ_{max} = 250 nm. Melting point: 197°C. Reacetylation yielded acetyl-11-keto-β-BA, identical with authentic sample.

α-BA and acetyl-α-BA. α-BA possess a geminal dimethyl group at C-20. The main characterization of the acids is carried out by noting the absence of the basepeak at m/z = 218 in the MS, which is characteristic of β-BA and its derivatives (Pardhy and Bhattacharyya, 1978). The main pattern of fragmentation of β-BA involving ring D and E units at m/z = 203 and 189 are also absent in α-BA (Hairfield *et al.*, 1989). The molecule peak, however, is identical with the data for β-BA and its acetyl derivative.

LT assay. LTB_4 formation, as well as the production of three diHETE isomers (6-*trans*-LTB_4, 12-epi-6-*trans*-LTB_4 and 5-(*S*), 12-(*S*)-diHETE) and 5-HETE in glycogen-elicited rat peritoneal PMNL by Ca^{++}-A 23187 stimulation were studied as described previously (Safayhi *et al.*, 1985). Briefly, 10^7 cells [5 × 10^6 cells/ml of buffer (in millimolar): NaCl, 137; KCl, 2.7; KH_2PO_4, 2; Na_2PO_4, 5; and glucose, 5, pH 7.2] were prewarmed for 5 min at 37°C. Thereafter, A23187 (1 μg/ml) was added and the mixture was incubated for a further 2 min. After adding the calcium solution to the final concentration of 1.75 mM, the mixture was incubated for a further 5 min at 37°C. To stop the reaction the cell suspension was acidified with ice-cold formic acid (1%) to pH 3. PGB_2 (300 ng) in MeOH was added to each sample as internal standard. Extraction was carried out with an equal volume of chloroform-MeOH (1:1). The extract was evaporated to dryness and resolved in MeOH for HPLC analysis. For the inhibition study the tested compounds dissolved in dimethylsulfoxide were added to the cells 2 min before the addition of A23187. All incubations including controls were carried out in the presence of dimethylsulfoxide (0.5%). Methanolic solution (20 μl) was injected *via* a Rheodyne 7125 device into a Shimadzu HPLC using detection wavelength of 280 nm in order to detect the internal standard PGB_2 and diHETEs or 235 nm in order to allow the detection of HETEs. Analysis was performed by isocratic elutions with MeOH-water-acetic acid = 72:28:0.2 or 67/33/0.2 (v/v), pH 4.8; flow rate: 1 ml/min.

12-LO and CO assay. 12-LO-catalyzed 12-HETE and CO-catalyzed 12-HHT formation from endogenous arachidonic acid were measured in washed human platelets. Blood samples were withdrawn from the arm veins of six volunteers into potassium-EDTA containing Monovette tubes (1.6 mg of EDTA per ml of blood). Erythrocytes were pelleted by centrifugating 5 ml of fresh blood samples after dilution with Ca^{++}-free buffer (1:2) at 150 g for 15 min. Four milliliters of buffer were added to 6 ml of the supernatant and centrifuged (150 × *g* for 15 min) to remove contaminating erythrocytes. Nine milliliters of the supernatant were then centrifuged for 12 min at 650 × *g* to collect highly enriched platelets. Samples of 8 × 10^7 platelets per ml were stimulated for 5 min in the presence or absence of test drugs with Ca^{++} and ionophore under conditions described above. Extraction after the addition of PGB_2 and analysis for 12-HHT and 12-HETE were carried out by rp-HPLC (Novo Pak column) and UV-detection (280 nm for 8 min and 235 nm). Mobile phase: MeOH-water-acetic acid = 67/33/0.2 (v/v), pH 4.8; flow rate: 1 ml/min.

Peroxidation assay. Arachidonic acid was peroxidized by a system described previously for liposomes (Ursini *et al.*, 1982): 0.1 M Tris-HCl, pH 7.5, 320 μg of arachidonic acid (sodium salt) dissolved in MeOH, 330 μM ascorbic acid and 5 μM $FeCl_3$ (added as a 1:10 Fe-ADP complex), in a volume of 3 ml. In modification, after incubation at 37°C for 30 min and the addition of 500 ng of PGB_2 as internal standard the acidified medium was extracted with one volume of MeOH-chloroform and analyzed for oxidized products by HPLC separation and UV-detection as described above and shown in figure 5.

Cell viability. Cell viability was assessed by the hemolytic activity with bovine erythrocytes by measuring hemoglobin in the supernatant at 540 nm and with PMNL by the trypan blue exclusion test.

Data. Percentage of inhibition was computed by comparing values in treatment groups to the value of individual controls. Data of independent observations (n = number of observations) are shown as means ± S.D.

Results

BAs are pentacyclic triterpene acids. The prominent form of BA from the gum resin exudate of *Boswellia serrata* is the β-isomer (fig. 1), beside the minor components α-BA and 11-keto-β-BA (see "Materials and Methods"). We could not isolate the 11-keto-derivative of the α-isomer probably due to scant presence of the α-form.

Figure 2 shows that acetyl-11-keto-β-BA inhibited the LTB_4 formation concentration dependently with an IC_{50} of 1.5 μM in Ca^{++}-ionophore-stimulated PMNL. All isolated forms of BA (acetylated, deacetylated and mixed forms) inhibited the 5-LO product formation with comparable IC_{50} being 1.5 μM (for acetyl-11-keto-β-BA), 4 to 5 μM (BA mixture, acetyl-BA mixture, 11-keto-β-BA, α-BA and β-BA) and 6.5 to 7 μM (acetyl-α-BA and acetyl-β-BA). All forms of BA decreased concentration dependently the *in vitro* synthesis of all 5-LO products from endogenous arachidonic acid (*i.e.*, 6-*trans*-LTB_4, 12-epi-6-*trans*-LTB_4, LTB_4, 5-(*S*),12-(*S*)-diHETE and 5-HETE) in parallel (data not shown). In contrast, β-GA, which is also a pentacyclic triterpene, did not substantially decrease the formation of LTB_4 and 5-HETE at comparable concentrations (for chemical structure see fig. 1). In our system the steroidal type anti-inflammatory drug hydrocortisone exerted no immediate effects in a concentration range from 10^{-12} to 10^{-5} M (results shown for 10^{-8} to 10^{-5} M in fig. 2). In our hands, the redox-type 5-LO inhibitor NDGA decreased the LTB_4 formation with an IC_{50} of 0.5 μM.

Figures 3 and 4 demonstrate that in washed human platelets NDGA inhibited the formation of the 12-LO product 12-HETE as well as the CO product 12-HHT with comparable IC_{50} values of 5 μM. In contrast, the BA in concentrations up to 400 μM exerted no significant effects on the 12-LO and CO activities *in vitro* (data shown for acetyl-BA in figs. 3 and 4). As expected, indomethacin decreased 12-HHT production in human platelets with an IC_{50} of 10 nm but increased 12-HETE formation in this system at comparable concentrations.

In a cell-free system the nonenzymatic peroxidation of arachidonic acid by Fe-ascorbate was almost abolished by 10 μM NDGA (fig. 5B). The IC_{50} for NDGA was about 5 μM and was

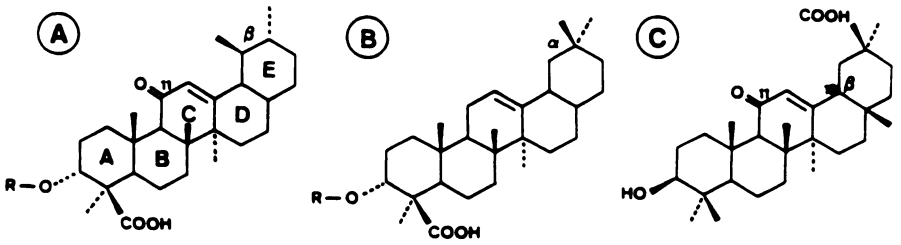

Fig. 1. Chemical structure of BAs and 18-β-GA. A, R = H, 11-keto-β-BA; R = CH_3CO-, acetyl-β-BA; B, R = H, α-BA; R = CH_3CO-, acetyl-α-BA; C, β-GA.

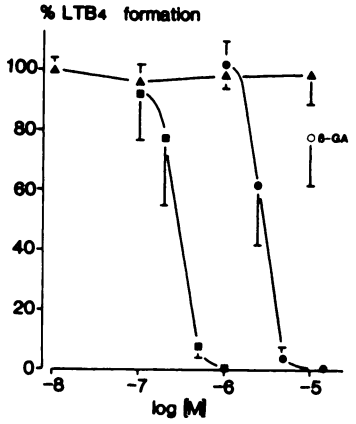

Fig. 2. Effects of acetyl-11-keto-β-BA (●), NDGA (■) and hydrocortisone (▲) on LTB_4 formation from endogenous arachidonic acid by Ca^{++}-ionophore-stimulated rat peritoneal PMNL. Data are in means ± S.D. as percentage of metabolite formation in controls; n = 3.

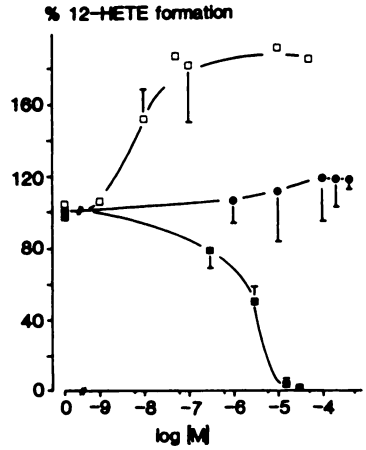

Fig. 3. Effects of acetyl-BA (●), NDGA (■) and indomethacin (□) on 12-HETE formation from endogenous arachidonic acid by Ca^{++}-ionophore-stimulated human platelets. Data are in means ± S.D. as percentage of 12-HETE formation in controls; n = 3.

in the same concentration range as observed for the effects on CO (12-HHT) and 12-LO (12-HETE) in the platelet test. In contrast, BA did not affect the chemical oxidation of arachidonic acid by Fe-ascorbate up to 400 μM (fig. 5A).

BA and acetyl-BA did not impair cell viability as assessed by the hemolytic activity with bovine erythrocytes and by the trypan blue exclusion test with PMNL. No substantial destabilization of erythrocyte integrity was observed up to 1 hr with 100 and 200 μM BA and acetyl-BA. In contrast, β-GA (100 and 200 μM) exerted cytolytic activity at 1 hr (91 ± 11% damaged erythrocytes within 1 hr by 200 μM β-GA; n = 3). No impairment of PMNL by BA and acetyl-BA up to 15 min was observed with trypan blue exclusion studies in incubations comparable to the conditions of the LT test except that no ionophore was added (data not shown).

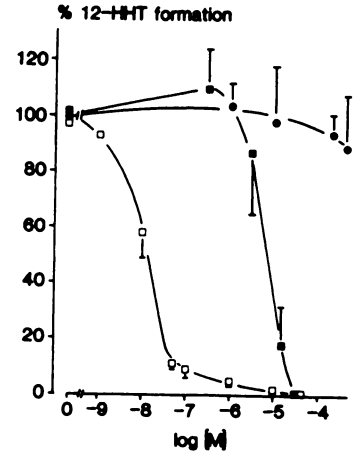

Fig. 4. Effects of acetyl-BA (●), NDGA (■) and indomethacin (□) on 12-HHT formation from endogenous arachidonic acid by Ca^{++}-ionophore-stimulated human platelets. Data are in means ± S.D. as percentage of 12-HHT formation in controls; n = 3.

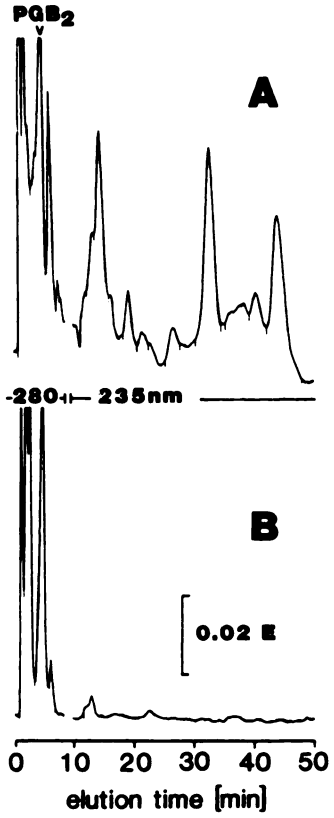

Fig. 5. Elution profiles of Fe-ascorbate-induced oxidation products from arachidonic acid in the presence of 400 μM acetyl-BA (A) and 10 μM NDGA (B).

Discussion

LTs, for which the 5-LO is the key enzyme, are considered to be involved in the initiation and the maintenance of a variety of inflammatory diseases, *e.g.*, chronic rheumatism, morbus Crohn, colitis ulcerosa, psoriasis, chronic asthma bronchitis and anaphylactic shock (for reviews see Samuelsson, 1983; Hammarström, 1983; Piper, 1984). From the family of LT-type mediators, LTB_4 is a stimulator of leukocyte responses like chemotaxis, cell adhesion, superoxide production, Ca^{++} translocation and release of hydrolytic enzymes (Ford-Hutchinson *et al.*, 1980; Malmsten *et al.*, 1980). Additionally, it stimulates pronounced plasma exudation *in vivo* (Bray *et al.*, 1981; Higgs *et al.*, 1981; Wedmore and Williams 1981). So far no products for specific 5-LO inhibition are available for therapeutic purposes.

Rat peritoneal PMNL, upon stimulation with Ca^{++}-ionophore, mainly produce LTB_4 and 5-HETE from endogenous arachidonic acid (Safayhi *et al.*, 1985). All isolated forms of BA concentration dependently reduced 5-LO product formation in PMNL with IC_{50} values from 1.5 to 7 μM. In comparison to two established 5-LO inhibitors (*i.e.*, NDGA and PZ51: Ebselen), in our test system BAs were less potent than NDGA but more effective than Ebselen, which were shown to decrease the glycogen-elicited, Ca^{++}-ionophore-stimulated rat neutrophil 5-LO activity in this system with an IC_{50} of 0.5 and 20 μM, respectively (Safayhi *et al.*, 1985). In this context it is noteworthy that IC_{50} data *in vitro* not only depend on the quality of the substance tested. For example, the inhibition of LTB_4 formation in human PMNL by MK866 was dependent upon cell concentration: A 10-fold increase in cell number (from 2.5×10^5 to 2×10^6 per assay) increased IC_{50} by about 20 times. Additionally, in glycogen-elicited PMNL the IC_{50} value for MK866 was roughly 3 times higher than with human blood PMNL (Gillard *et al.*, 1989). Our present tests were carried out with 10^7 glycogen-elicited rat peritoneal PMNL per assay.

At all tested concentrations the BAs decreased LTB_4, its all-*trans*-isomers and 5-HETE levels in parallel. Therefore, an effect of BAs on the LTA_4 hydrolase and/or a glutathione peroxidase mimicking activity are unlikely. Because no inhibition of the 12-HHT production (CO pathway) and 12-HETE formation (12-LO pathway) by BA up to 400 μM from endogenous arachidonic acid in platelets was observed, it is likely that the activity of BA is selective for the 5-LO pathway of arachidonic acid metabolism. Because, upon Ca^{++}-ionophore stimulation, neither the formation of 12-HHT nor the production of 12-HETE from endogenous arachidonic acid were affected by BAs, we suggest that the phospholipase A_2 activity is not impaired by BAs. Furthermore, from these observations in addition to the lack of an effect on Fe-ascorbate oxidation of arachidonic acid we deduce that the mechanism of the inhibition by BA is not a nonselective reduction. From the present data it cannot be decided whether BA-like MK866-act as "translocation inhibitors" by an interaction with the 5-LO-activating protein (Miller *et al.*, 1990; Dixon *et al.*, 1990) or directly inhibit the 5-LO.

Therefore, in conclusion, the data of the present study suggest that BAs are novel and highly specific inhibitors of the 5-LO. In contrast of the large number of existing "antioxidant-type" 5-LO inhibitors (NDGA, BW755c, quercetin, hydroxyquinoline, caffeic acid, etc.), BAs do not act by a reduction of the peroxide tonus and, therefore, do not decrease the activities of 12-LO and CO.

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Send reprint requests to: Dr. Hasan Safayhi, Department of Pharmacology, Pharmaceutical Institute, University of Tuebingen, Auf der Morgenstelle 8, 7400 Tuebingen, FRG.