



## Immunopharmacology and Inflammation

On the interference of boswellic acids with 5-lipoxygenase: Mechanistic studies *in vitro* and pharmacological relevanceUlf Siemoneit<sup>a</sup>, Carlo Pergola<sup>a</sup>, Bianca Jazsar<sup>a</sup>, Hinnak Northhoff<sup>b</sup>, Carsten Skarke<sup>c</sup>, Johann Jauch<sup>d</sup>, Oliver Werz<sup>a,\*</sup><sup>a</sup> Department for Pharmaceutical Analytics, Pharmaceutical Institute, University of Tuebingen, Auf der Morgenstelle 8, D-72076 Tuebingen, Germany<sup>b</sup> Institute for Clinical and Experimental Transfusion Medicine, University Medical Center Tuebingen, Hoppe-Seyler-Straße 3, 72076 Tuebingen, Germany<sup>c</sup> Pharmazentrum Frankfurt, ZAFES, Institut für Klinische Pharmakologie, Klinikum der Johann Wolfgang Goethe-Universität, Frankfurt, Germany<sup>d</sup> Institute of Organic Chemistry, University of Saarland, Im Stadtwald 23.2, D-66041 Saarbrücken, Germany

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## ABSTRACT

Boswellic acids are pharmacologically active ingredients of frankincense with anti-inflammatory properties. It was shown that *in vitro* 11-keto-boswellic acids inhibit 5-lipoxygenase (5-LO, EC 1.13.11.34), the key enzyme in leukotriene biosynthesis, which may account for their anti-inflammatory effectiveness. However, whether 11-keto-boswellic acids interfere with 5-LO under physiologically relevant conditions (i.e., in whole blood assays) and whether they inhibit 5-LO *in vivo* is unknown. Inhibition of human 5-LO by the major naturally occurring boswellic acids was analyzed in cell-free and cell-based activity assays. Moreover, interference of boswellic acids with 5-LO in neutrophil incubations in the presence of albumin and in human whole blood was assessed, and plasma leukotriene B<sub>4</sub> of frankincense-treated healthy volunteers was determined. Factors influencing 5-LO activity (i.e., Ca<sup>2+</sup>, phospholipids, substrate concentration) significantly modulate the potency of 11-keto-boswellic acids to inhibit 5-LO. Moreover, 11-keto-boswellic acids efficiently suppressed 5-LO product formation in isolated neutrophils (IC<sub>50</sub>=2.8 to 8.8 μM) but failed to inhibit 5-LO product formation in human whole blood. In the presence of albumin (10 mg/ml), 5-LO inhibition by 11-keto-boswellic acids (up to 30 μM) in neutrophils was abolished, apparently due to strong albumin-binding (>95%) of 11-keto-boswellic acids. Finally, single dose (800 mg) oral administration of frankincense extracts to human healthy volunteers failed to suppress leukotriene B<sub>4</sub> plasma levels. Our data show that boswellic acids are direct 5-LO inhibitors that efficiently suppress 5-LO product synthesis in common *in vitro* test models, however, the pharmacological relevance of such interference *in vivo* seems questionable.

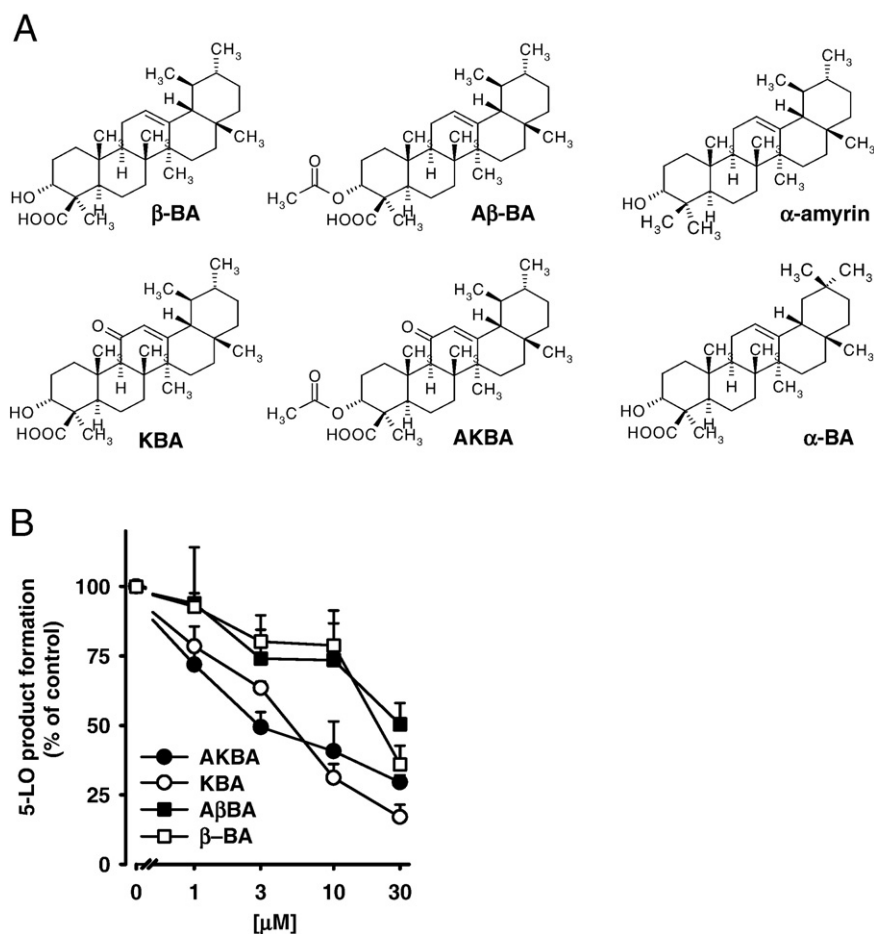
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## 1. Introduction

Frankincense extracts are applied in folk medicine to cure chronic inflammatory diseases, and experimental data from animal models and studies with human subjects indicate a therapeutic value of these remedies in the treatment of inflammatory and allergic disorders (Ammon, 2006; Poeckel and Werz, 2006). Clinical trials with pilot character indicate beneficial effects of frankincense preparations in osteoarthritis (Sengupta et al., 2008), Crohn Disease, and collagenous colitis (Ammon, 2006; Madisch et al., 2007). Frankincense extracts were found to inhibit leukotriene B<sub>4</sub> formation in activated rat polymorphonuclear leukocytes (Ammon et al., 1991) and also the major ingredients of these extracts, the pentacyclic triterpenes boswellic acids (Fig. 1A), blocked leukotriene formation in this assay (Safayhi et al., 1992). Since

boswellic acids interfere with 5-lipoxygenase (5-LO), the key enzyme in leukotriene biosynthesis (Safayhi et al., 1995; Sailer et al., 1998, 1996; Werz et al., 1997), a molecular mechanisms underlying the anti-inflammatory effectiveness of frankincense preparations was proposed. Among the four major β-configured boswellic acids from frankincense, the 3-O-acetyl-11-keto-boswellic acid (AKBA) is the most potent analogue with IC<sub>50</sub> values in the range of 1.5 to 50 μM, depending on the assay conditions (e.g. species, cell type, cell-free or cell-based assays, stimuli etc.; reviewed by Poeckel and Werz, 2006). AKBA seemingly belongs to the class of nonredox-type 5-LO inhibitors (Safayhi et al., 1995), and interferes in the presence of Ca<sup>2+</sup> with a fatty acid-binding site of 5-LO which is distinct from the substrate binding cleft (Sailer et al., 1998). In addition to 5-LO, AKBA also inhibits related enzymes within the AA cascade, including platelet-type 12-LO (IC<sub>50</sub>=17 μM, EC 1.13.11.31) (Poeckel et al., 2006) and cyclooxygenase-1 (IC<sub>50</sub>=6–32 μM, EC 1.14.99.1) (Siemoneit et al., 2008) as well as cytochrome P450 enzymes (IC<sub>50</sub>=5–10 μM) (Frank and Unger, 2006), and suppresses the activity of human leukocyte elastase (HLE, IC<sub>50</sub>=15 μM, EC 3.4.21.37) (Safayhi et al., 1997),

\* Corresponding author. Tel.: +49 70712978793; fax: +49 7071294565.  
E-mail address: [oliver.werz@uni-tuebingen.de](mailto:oliver.werz@uni-tuebingen.de) (O. Werz).



**Fig. 1.** Chemical structures of various boswellic acids and  $\alpha$ -amyrin and inhibition of 5-LO. (A) Chemical structures:  $\alpha$ -BA ( $\alpha$ -boswellic acid), A $\beta$ -BA (3-O-acetyl- $\beta$ -boswellic acid); AKBA (3-O-acetyl-11-keto- $\beta$ -boswellic acid);  $\beta$ -BA ( $\beta$ -boswellic acid); KBA (11-keto- $\beta$ -boswellic acid). (B) Inhibition of human 5-LO. Partially purified, recombinant, human 5-LO (0.5  $\mu$ g) expressed in *E. coli* was pre-incubated in 1 ml PBS pH 7.4 containing 1 mM EDTA and 1 mM ATP with the indicated concentrations of boswellic acids or vehicle (DMSO) at 4 °C. After 10 min, samples were pre-warmed at 37 °C for 30 s and 5-LO product formation was started by addition of 2 mM CaCl<sub>2</sub> and 20  $\mu$ M AA. After another 10 min, 5-LO product formation was determined. Values are given as mean  $\pm$  S.E.M., n=3–4.

of topoisomerases (IC<sub>50</sub>=10–30  $\mu$ M; EC 5.99.1.2/3) (Syrovets et al., 2000), and of I $\kappa$ B kinases (IC<sub>50</sub>=1–10  $\mu$ M, EC 2.7.11.10) (Syrovets et al., 2005).

The pharmacological relevance of the putative targets of AKBA *in vivo* is unclear. Most of these interactions are observed at relatively high concentrations *in vitro* (IC<sub>50</sub>=1 to 50  $\mu$ M) (PoECKel and Werz, 2006) which are far above the plasma levels of AKBA (<0.1  $\mu$ M) reached after oral application of standard dosage of frankincense extracts (Buchele and Simmet, 2003). Moreover, the pathophysiological roles of the proposed targets of boswellic acids, often do not clearly concur with the beneficial effects of frankincense extracts seen in animal models or in patients. For example, 5-LO and leukotrienes play established roles in asthma and allergic rhinitis, whereas the connection to osteoarthritis and chronic inflammatory bowel disease, where frankincense showed efficacy in human patients, is unlikely (Peters-Golden and Henderson, 2007; Werz and Steinhilber, 2006). Finally, suppression of leukotriene formation by boswellic acids or frankincense extracts was analysed so far only in cell-free assays or in isolated leukocytes from rats or guinea pigs but never in a physiological context (e.g. human whole blood assays or in humans *in vivo*) or under experimental conditions that consider pharmacologically relevant factors (i.e. plasma albumin-binding). Hence, the pharmacological relevance of the inhibition of 5-LO by boswellic acids *in vivo* is unclear. Here we provide a detailed evaluation of the effectiveness of boswellic acids to inhibit 5-LO product formation in selected test systems, taking into account essential parameters that affect the efficacy *in vivo*.

## 2. Materials and methods

### 2.1. Materials

Boswellic acids were prepared as described previously (Jauch and Bergmann, 2003).  $\alpha$ -Amyrin was from Extrasynthèse (Genay, France); *Boswellia serrata* resin extract (H15® Gufic) was from Gufic Chem (Belgaum, Karnataka, India); N-(3-phenoxyacetyl) acetoxyhydroxamic acid (BWA4C) was a generous gift by Dr. L. G. Garland (Wellcome Research Laboratories, London, UK). Adenosine-5'-triphosphate (ATP) was from Roche Diagnostics (Mannheim, Germany) phosphatidylcholine, N-formyl-methionyl-leucyl-phenylalanine (fMLP), lipopolysaccharide (LPS), fatty acid-free bovine serum albumin (BSA) and all other fine chemicals were obtained by Sigma (Deisenhofen, Germany), unless stated otherwise.

### 2.2. Cells

Neutrophils were freshly isolated from human venous blood of healthy adult donors (Blood Center, University Hospital, Tuebingen, Germany) as described (Werz et al., 2002). In brief, venous blood was taken from donors and leukocyte concentrates were prepared by centrifugation at 4000  $\times$ g, 20 min, 20 °C. Neutrophils were immediately isolated by dextran sedimentation, centrifugation on Nycoprep cushions, and hypotonic lysis of erythrocytes. Neutrophils (5  $\times$  10<sup>6</sup> cells/ml; purity >96–97%) were finally resuspended in phosphate-buffered saline

pH 7.4 (PBS) plus 1 mg/ml glucose (PG buffer) or in PG buffer plus 1 mM CaCl<sub>2</sub> (PGC buffer) as indicated. For incubations with solubilized compounds, DMSO was used as vehicle, never exceeding 0.2% (v, v). To exclude toxic effects of boswellic acids during pre-incubation periods, neutrophil viability was analysed by light microscopy and trypan blue exclusion. Incubation with 30 μM of any of the boswellic acids at 37 °C for up to 30 min caused no significant change in neutrophil viability.

### 2.3. Expression of human recombinant 5-LO in *E. coli* and preparation of high speed supernatants

*E. coli* MV1190 was transformed with pT3-5LO plasmid, and recombinant 5-LO protein was expressed at 30 °C as described (Fischer et al., 2003). Cells were lysed by incubation in 50 mM triethanolamine-HCl pH 8.0, 5 mM EDTA, soybean trypsin inhibitor (60 μg/ml), 1 mM phenylmethanesulphonyl fluoride (PMSF), 2 mM dithiothreitol, and lysozyme (1 mg/ml), homogenized by sonication (3 × 15 s) and centrifuged at 40,000 ×g for 20 min at 4 °C. The resulting 40,000 ×g supernatant was immediately used for 5-LO activity assays.

### 2.4. Determination of 5-LO product synthesis in human neutrophils

For determination of cellular 5-LO product formation, 5 × 10<sup>6</sup> freshly isolated neutrophils in 1 ml PGC buffer with or without BSA were pre-incubated with test compounds or with vehicle (DMSO) for 10 min at 37 °C, as indicated. 5-LO product formation was started by addition of A23187 (2.5 μM) with or without 20 μM AA. The reaction was stopped after 10 min with 1 ml of methanol and 30 μl of 1 N HCl, 200 ng prostaglandin B<sub>1</sub> and 500 μl of PBS were added. Formed 5-LO metabolites were extracted and analyzed by HPLC as described (Werz et al., 2002). 5-LO product formation is expressed as ng of 5-LO products per 10<sup>6</sup> cells which includes leukotriene B<sub>4</sub> and its all-trans isomers, 5(S),12(S)-di-hydroxy-6,10-trans-8,14-cis-eicosatetraenoic acid (5(S),12(S)-DiHETE), and 5-H(P)ETE. 5-HETE and 5-HPETE coelute as one major peak, integration of this peak represents both eicosanoids. Cysteinyl leukotrienes C<sub>4</sub>, D<sub>4</sub> and E<sub>4</sub> were not detected and oxidation products of leukotriene B<sub>4</sub> were not determined.

### 2.5. Determination of 5-LO product synthesis in human whole blood

For assays in whole blood, freshly withdrawn blood from healthy adult donors was obtained by venipuncture and collected in monovettes containing 16 IE heparin/ml. Aliquots of 2 ml (A23187) or 3 ml (LPS and fMLP) were pre-incubated with the test compounds or with vehicle (DMSO) for 10 min at 37 °C, as indicated, and formation of 5-LO products was either started by addition of fMLP (1 μM) following priming of blood with 1 μg/ml LPS for 30 min, or by addition of A23187 (30 μM). The reaction was stopped on ice after 15 (LPS priming and stimulation with fMLP) or 10 (stimulation with A23187) min and the samples were centrifuged (600 ×g, 10 min, 4 °C). Aliquots of the resulting plasma (500 μl) were then mixed with 2 ml of methanol and 200 ng prostaglandin B<sub>1</sub> were added as internal standard. The samples were placed at -20 °C for 2 h and centrifuged again (600 ×g, 15 min, 4 °C). The supernatants were collected and diluted with 2.5 ml PBS and 75 μl HCl 1 N. Formed 5-LO metabolites were extracted and analyzed by HPLC as described for intact neutrophils.

### 2.6. Determination of 5-LO product synthesis in 40,000 g supernatants of *E. coli* and of partially purified 5-LO

Aliquots of the supernatants of *E. coli* lysates (corresponding to 4 ml *E. coli* culture) or 0.5 μg partially purified recombinant 5-LO, were diluted with ice-cold PBS containing 1 mM EDTA, and 1 mM ATP and phosphatidylcholine as well as the test compounds (or DMSO as vehicle) were added, as indicated. After 10 min on ice, samples were pre-warmed for 30 s at 37 °C and AA (2 or 20 μM) was added together

with or without 2 mM CaCl<sub>2</sub>, as indicated. The reaction was stopped after 10 min at 37 °C by addition of 1 ml of ice-cold methanol and 200 ng prostaglandin B<sub>1</sub> were added. Formed metabolites were extracted and analyzed by HPLC as described above for neutrophils.

### 2.7. Determination of leukotriene B<sub>4</sub> formation in plasma of human subjects and clinical study protocol

In a phase 1 clinical trial the safety, tolerability, and pharmacokinetics of single dose cross over application of two *B. serrata* resin extract PS0201Bo soft gelatine capsules in healthy male volunteers was investigated. Two capsules of PS0201Bo each containing 400 mg of native *B. serrata* resin extract (total 800 mg PS0201Bo) were taken by healthy adult male volunteers in the morning immediately after fasting and after a defined meal. Venous blood samples were collected from the volunteers after 0, 2, 4, 8, and 24 h, citrated and plasma was prepared. Aliquots of the plasma were immediately used for determination of leukotriene B<sub>4</sub> by an ELISA according to the instructions of the manufacturer (IBL, Hamburg, Germany).

The study protocol and a sample volunteer information and consent form were reviewed by the competent Ethics Committee (IECs) of the University of Frankfurt, Germany, and a favourable opinion was issued on July 6, 2006; regulatory authority approvals were obtained from the competent Higher Federal Authority, the Bundesinstitut für Arzneimittel und Medizinprodukte (BfArM), Bonn, Germany (EudraCT no: 2006-002939-24). This trial was conducted in compliance with the protocol, principles of the Declaration of Helsinki (1996), and the ICH GCP guideline (manuscript in preparation).

### 2.8. Albumin-binding of boswellic acids

PG buffer (2.5 ml), supplemented with BSA (0, 1 or 10 mg/ml, as indicated) was incubated with 50 μM AKBA for 10 min at 37 °C. Separation of AKBA and albumin was carried out by gel filtration using a Sephadex® G-25 (PD-10) column (GE Healthcare, Freiburg, Germany). In brief, the Sephadex® G-25 column was equilibrated with PG buffer and the sample (2.5 ml) was applied. 500 μl aliquots of the eluate were collected and immediately analysed for AKBA by HPLC, and for BSA using Roti®-nanoquant according to the instructions of the manufacturer (Carl Roth, Karlsruhe, Germany). Quantification of AKBA by RP-HPLC was performed on a Nova-Pak® C18 column (5 × 100 mm, 4 μm particle size, Waters (Eschborn, Germany)) using 85% methanol aq. + 0.01% TFA (v, v) as mobile phase at a flow rate of 1.2 ml/min and UV detection at 250 nm. Calibration was based on the external standard method, with the peak area as assay parameter. The concentration of AKBA (without BSA) versus elution volume was analysed by a non-linear fit (Gaussian distribution) providing μ, δ and the area under the curve (AUC) using the GraphPad Prism 4 (GraphPad software Inc., San Diego, CA, USA) program. Based on this evaluation the amount of unbound AKBA was determined by the AUC (μ ± 3δ) for incubation with 1 or 10 mg/ml BSA.

**Table 1**

Efficacy of various boswellic acids for inhibition of 5-LO in different test systems

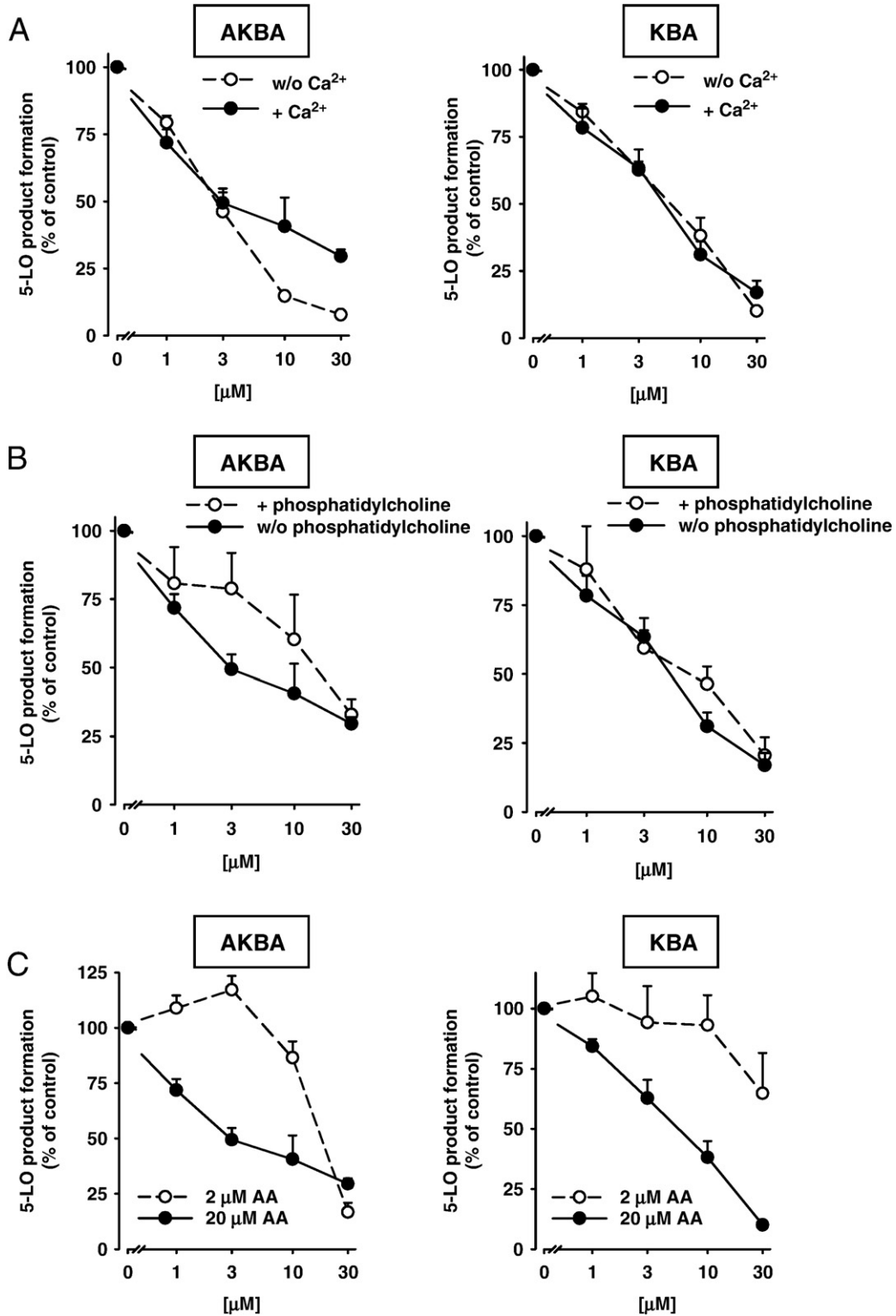
Compound	5-LO purified enzyme	Supernatants	Neutrophils	
			(A23187)	(A23187 + AA)
AKBA	2.9	23.5	3.2	3.3
KBA	6.3	27.1	8.8	2.8
Αβ-BA	>30	>30	>30	>30
β-BA	23.9	>30	>30	28.1
α-BA	15.3	18.5	23.1	16
α-amyrin	>>30	>>30	>>30	>>30
BWA4C	0.10	0.15	0.03	0.05

IC<sub>50</sub> values (μM) of boswellic acids were determined regarding 5-LO activity of human purified 5-LO enzyme, crude 5-LO in supernatants of *E. coli* lysates (standard activity assay in the presence of 2 mM Ca<sup>2+</sup> and 20 μM AA), and in isolated human neutrophils stimulated with either 2.5 μM A23187 with or without 20 μM AA. The synthetic 5-LO inhibitor BWA4C was used as control.

2.9. Statistics

Data are expressed as mean±S.E.M.. IC<sub>50</sub> values, obtained from measurements at 4–5 different concentrations of the compounds,

were calculated by GraphPad Prism 4 and data fit was obtained using the sigmoidal-concentration response equation (variable slope). Statistical evaluation of the data was performed by one-way ANOVAs for independent or correlated samples followed by Tukey HSD post-



**Fig. 2.** Modulation of inhibition of human 5-LO by boswellic acids in cell-free assays. (A) Effect of Ca<sup>2+</sup>. Partially purified, recombinant, human 5-LO (0.5 μg) expressed in *E. coli* was pre-incubated in 1 ml PBS pH 7.4 containing 1 mM EDTA and 1 mM ATP with the indicated concentrations of AKBA (left panel) or KBA (right panel) or vehicle (DMSO) at 4 °C. After 10 min, samples were prewarmed at 37 °C for 30 s, 2 mM CaCl<sub>2</sub> (as indicated) and 20 μM AA was added and after another 10 min, 5-LO product formation was determined. (B) Effect of phosphatidylcholine. Partially purified 5-LO (0.5 μg) was pre-incubated as in the standard assay together with or without 25 μg/ml phosphatidylcholine. Samples were pre-warmed at 37 °C for 30 sec, 2 mM CaCl<sub>2</sub> and 20 μM AA was added and after 10 min 5-LO product formation was determined. (C) Inhibition of 5-LO by boswellic acids at 2 and 20 μM AA. Partially purified 5-LO (0.5 μg) was pre-incubated with the test compounds (see (A)). Samples were pre-warmed at 37 °C for 30 sec, 2 mM CaCl<sub>2</sub> and AA (2 or 20 μM, as indicated) was added and after another 10 min, 5-LO product formation was determined. Values are given as mean±S.E.M., n=3–4.

hoc tests (GraphPad Software Inc.). Where appropriate, Student's *t* test for paired and correlated samples was applied. A *P* value <0.05 was considered significant.

### 3. Results

#### 3.1. Inhibition of 5-LO activity by boswellic acids in cell-free assays and influence of assay parameters on the potency

The efficacy of 5-LO inhibitors, in intact cells as well as in cell-free assays, depends on the assay conditions such as the substrate concentration, presence of 5-LO stimulatory co-factors (e.g. phosphatidylcholine, lipid hydroperoxides,  $\text{Ca}^{2+}$ ) and stimuli used to challenge intact cells (for review see Werz, 2007; Werz and Steinhilber, 2005). Previous studies demonstrated that partially purified 5-LO from human neutrophils is inhibited by AKBA with an  $\text{IC}_{50}$  value of 16  $\mu\text{M}$  (Safayhi et al., 1995). Other boswellic acids were not tested. Therefore, we first analyzed the potency of natural occurring boswellic acids to inhibit partially purified human recombinant 5-LO from *E. coli* in a well-established and defined cell-free assay using 20  $\mu\text{M}$  AA as substrate and 1 mM  $\text{Ca}^{2+}$  as supplement (termed standard activity assay) (Burkert et al., 2003). The major  $\beta$ -configured boswellic acids AKBA, 11-keto-boswellic acid (KBA),  $\beta$ -boswellic acid ( $\beta$ -BA), and acetyl- $\beta$ -boswellic acid ( $\text{A}\beta$ -BA) inhibited 5-LO product synthesis in a concentration-dependent manner, with  $\text{IC}_{50}$  values of 2.9, 6.3, 23.9, and 30  $\mu\text{M}$ , respectively (Fig. 1B, Table 1). Of interest, also the  $\alpha$ -boswellic acid ( $\alpha$ -BA) inhibited 5-LO activity with an  $\text{IC}_{50}$  = 15.3  $\mu\text{M}$  (Table 1), whereas the pentacyclic triterpene  $\alpha$ -amyrin (lacking the essential C4-carboxylic group) failed in this respect ( $\text{IC}_{50}$  >> 30  $\mu\text{M}$ ).

Subsequent studies under selected assay conditions were carried out with the most efficient derivatives, i.e. AKBA and KBA. As observed before (Burkert et al., 2003), removal of  $\text{Ca}^{2+}$  from the standard activity assay increased 5-LO activity under these incubation conditions about two-fold, but the efficacies of KBA and AKBA were not markedly changed (Fig. 2A, Table 2). The efficacy of AKBA at higher concentrations (10 and 30  $\mu\text{M}$ ) was slightly increased in the absence of  $\text{Ca}^{2+}$ . The presence of 25  $\mu\text{g}/\text{ml}$  phosphatidylcholine in the standard activity assay, often included as stimulatory factor in 5-LO activity assays (Rådmark et al., 2007; Reddy et al., 2000), impaired the 5-LO inhibitory potential of AKBA ( $\text{IC}_{50}$  = 19.8  $\mu\text{M}$ ), whereas the potency of KBA was essentially unaffected (Fig. 2B, Table 2). Of interest, switching from 20 to 2  $\mu\text{M}$  AA as 5-LO substrate in the standard activity assay significantly reduced the potency of both 11-keto-boswellic acids, and the  $\text{IC}_{50}$  values were determined at 21.5  $\mu\text{M}$  for AKBA and >30  $\mu\text{M}$  for KBA (Fig. 2C, Table 2). A weak loss of potency was observed also for BWA4C, a well-recognized iron ligand-type 5-LO inhibitor.

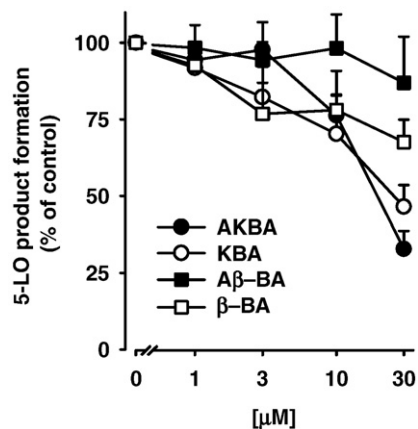
Finally, when 40,000  $\times\text{g}$  supernatants of *E. coli* lysates were used as source of crude 5-LO enzyme in the standard activity assay, the potency of all four  $\beta$ -configured boswellic acids was strongly impaired (Fig. 3, Table 1). Note that  $\alpha$ -BA hardly lost its potency and was the most potent derivative with  $\text{IC}_{50}$  = 18.5  $\mu\text{M}$  (Table 1). Taken together, both  $\alpha$ - and  $\beta$ -configured boswellic acids act as direct 5-LO inhibitors in cell-free assays, but the potencies strongly depend on the defined assay conditions, namely factors regulating 5-LO catalysis.

**Table 2**

Influence of assay parameters on the potencies of AKBA and KBA to inhibit 5-LO

Compound	AA (20 $\mu\text{M}$ ) –	AA (20 $\mu\text{M}$ ) + $\text{Ca}^{2+}$	AA (2 $\mu\text{M}$ ) + $\text{Ca}^{2+}$	AA (20 $\mu\text{M}$ ) + $\text{Ca}^{2+}$ + phosphatidylcholine
AKBA	2.8	2.9	11.5	19.8
KBA	7.6	6.3	>30	11.4
BWA4C	0.14	0.10	0.24	0.11

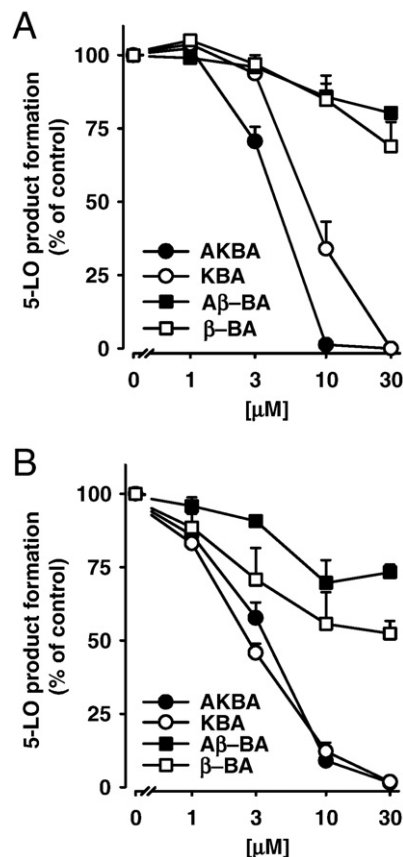
$\text{IC}_{50}$  values ( $\mu\text{M}$ ) for AKBA and KBA assessing the activity of partially purified 5-LO (0.5  $\mu\text{g}$ ) in the presence or absence of 1 mM free  $\text{Ca}^{2+}$ . Phosphatidylcholine (25  $\mu\text{g}/\text{ml}$ ) and AA (2 or 20  $\mu\text{M}$ ) were added as indicated. The synthetic 5-LO inhibitor BWA4C was used as control.



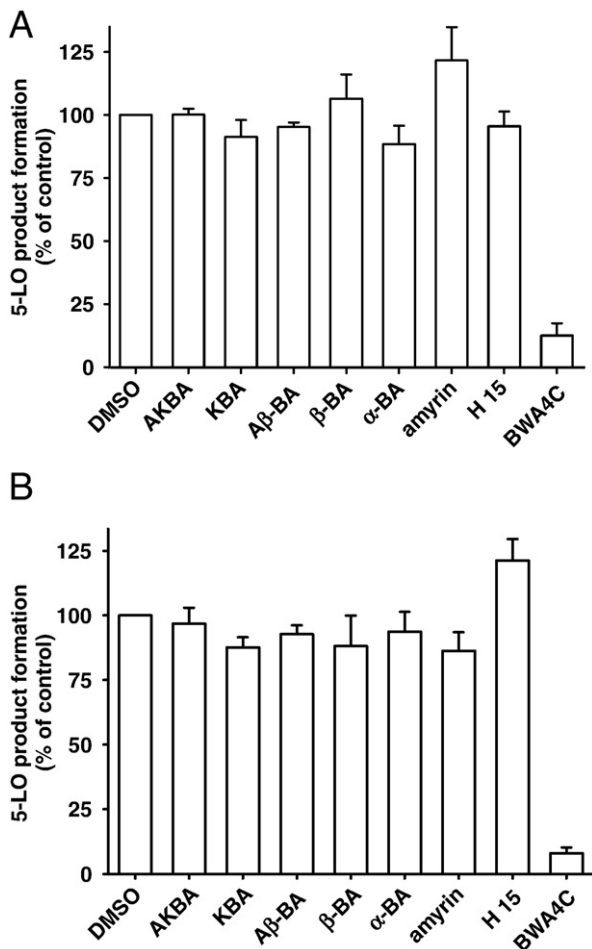
**Fig. 3.** Effects of boswellic acids on crude 5-LO in supernatants from *E. coli* lysates. Supernatants (40,000  $\times\text{g}$ ) from *E. coli* lysates (in PBS containing 1 mM EDTA, 1 mM PMSF, and 1 mM ATP) were preincubated with the test compounds at 4 °C. After 10 min samples were pre-warmed at 37 °C for 30 s, 2 mM  $\text{CaCl}_2$  and 20  $\mu\text{M}$  AA was added and after 10 min 5-LO product formation was determined. Values are given as mean  $\pm$  S.E.M.,  $n$  = 3–4.

#### 3.2. Inhibition of 5-LO activity by boswellic acids in cell-based assays using isolated human resuspended neutrophils

Neutrophils stimulated with ionophore A23187 are the classical test system for screening and evaluation of leukotriene synthesis



**Fig. 4.** Inhibition of 5-LO product formation in intact human neutrophils. Freshly isolated neutrophils ( $5 \times 10^6$ ) were resuspended in 1 ml PGC and test compounds or vehicle (DMSO) were added at the indicated concentrations. After 10 min at 37 °C, cells were stimulated with 2.5  $\mu\text{M}$  A23187 (A) or with 2.5  $\mu\text{M}$  A23187 plus 20  $\mu\text{M}$  AA (B), and 5-LO products were extracted and determined by HPLC. 5-LO product synthesis in the absence of test compounds (100%, control) was  $68.1 \pm 6$  (A23187) and  $276.8 \pm 40$  (A23187 + AA) ng per  $10^6$  neutrophils. Values are given as mean  $\pm$  S.E.M.,  $n$  = 3.



**Fig. 5.** Inhibition of 5-LO product formation in human whole blood. Freshly withdrawn blood 2 ml (A23187) and 3 ml (LPS and fMLP) was pre-incubated with test compounds (50  $\mu$ M, each, except BWA4C, 3  $\mu$ M) or vehicle (DMSO) at 37 °C, and formation of 5-LO products was started by addition of (A) A23187 (30  $\mu$ M; 10 min, 37 °C) or by (B) fMLP (1  $\mu$ M; 15 min, 37 °C) after 30 min priming with LPS (1  $\mu$ g/ml). The formation of 5-LO products (leukotriene B<sub>4</sub> and its all-trans isomers, 5 (S),12(S)-DiHETE), and 5-H(P)ETE) were determined as described. 5-LO product synthesis in the absence of test compounds (100%, control) was 196.9  $\pm$  46 (A23187) and 34.3  $\pm$  0.5 (LPS and fMLP) ng/ml blood. Data are mean  $\pm$  S.E.M.; n = 3–4 (fMLP and LPS), duplicates; n = 3 (A23187).

inhibitors, including compounds that act on AA release (cPLA<sub>2</sub> inhibitors), 5-LO, or 5-LO-activating protein (Werz, 2007; Werz and Steinhilber, 2005). By supplementation of exogenous AA, the possibility is given to circumvent PLA<sub>2</sub> inhibition and to estimate FLAP (an AA-binding protein that facilitates AA transfer to 5-LO) interference (Fischer et al., 2007; Werz, 2007). In agreement with previous studies using rat neutrophils (Safayhi et al., 1992, 1995; Sailer et al., 1996), AKBA and KBA potently inhibited 5-LO product synthesis in human isolated neutrophils challenged by A23187 in a concentration-dependent manner with IC<sub>50</sub> = 3.2 and 8.8  $\mu$ M, respectively (Fig. 4A, Table 1), and also the frankincense extract H15® blocked 5-LO product synthesis by 78  $\pm$  9% at a concentration of 30  $\mu$ g/ml. Aβ-BA, β-BA, and α-amyirin were hardly active (IC<sub>50</sub> > 30  $\mu$ M), but α-BA suppressed 5-LO product synthesis with an IC<sub>50</sub> = 23.1  $\mu$ M (Table 1). Upon stimulation of neutrophils with A23187 plus exogenous AA (20  $\mu$ M), the efficacy of AKBA was not altered, whereas the potency of KBA increased (IC<sub>50</sub> = 2.8) and also α-BA was more active under these conditions (IC<sub>50</sub> = 16  $\mu$ M) (Table 1). From these results one may conclude that AKBA and KBA are potent suppressors of 5-LO product formation in human neutrophils, essentially independent of the substrate (AA) supply, with similar efficacy as initially described for neutrophils from rats (Safayhi et al., 1995; Sailer et al., 1996).

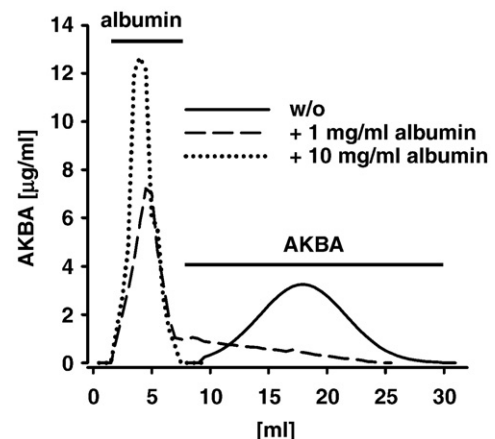
### 3.3. Inhibition of 5-LO activity by boswellic acids in human whole blood

Several inhibitors of leukotriene biosynthesis proved highly potent in isolated leukocytes but failed to efficiently interfere with 5-LO product formation in whole blood, due to plasma protein binding or competition with endogenous blood components such as fatty acids (Werz, 2002). However, the ability of a given compound to suppress leukotriene formation in the whole blood assay adequately reflects its efficacy *in vivo*. Human whole blood was pre-incubated with the test compounds (10 min) and stimulated either with A23187 or primed with LPS and then challenged with fMLP. In contrast to activation by the ionophore A23187, stimulation of blood with fMLP following LPS-priming is considered a physiologically relevant stimulus, thus, reflecting pathophysiological conditions *in vivo*. As shown in Fig. 5, despite the potent inhibition of 5-LO activity in cell-free assays or in intact neutrophils, neither the boswellic acids (50  $\mu$ M) nor the frankincense extract H 15® (30  $\mu$ g/ml) suppressed 5-LO product formation. The control inhibitor BWA4C efficiently inhibited 5-LO activity in the whole blood assay as expected.

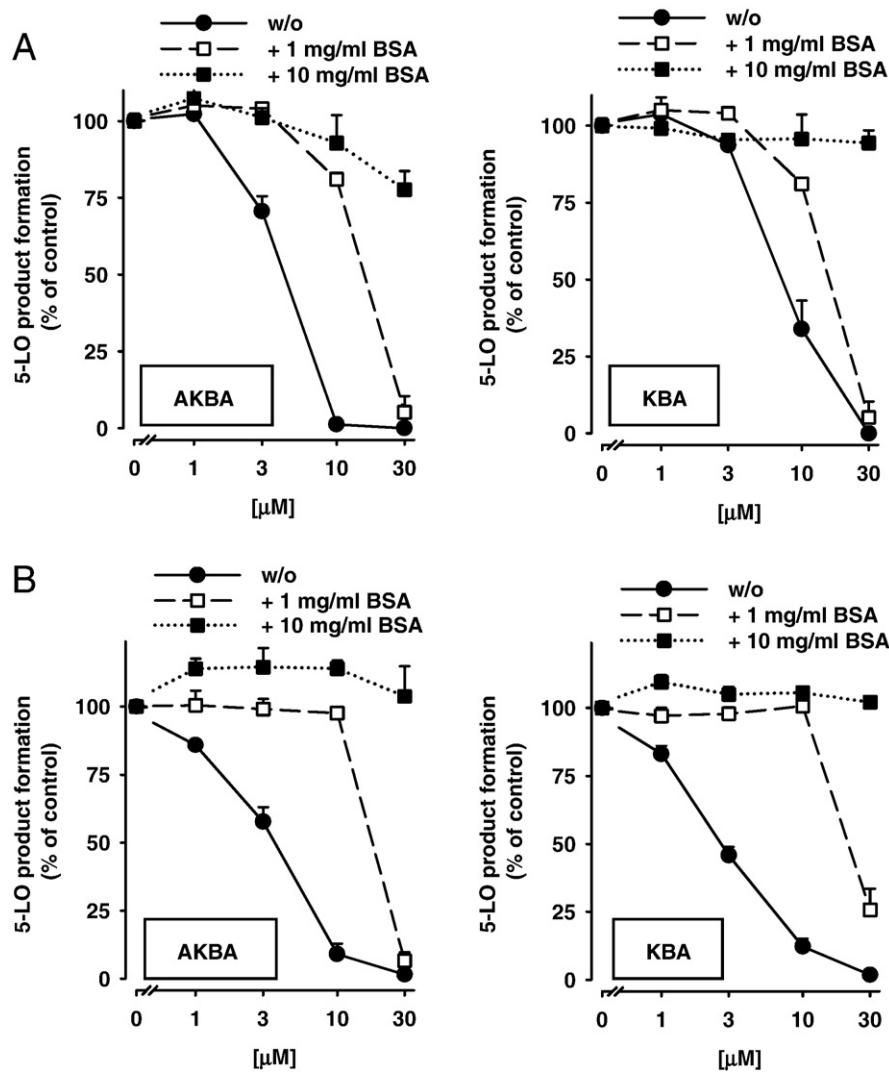
### 3.4. Inhibition of 5-LO activity by boswellic acids in human isolated neutrophils is abolished by albumin

Since AKBA and KBA are inactive in whole blood while being efficient in isolated neutrophils, and because boswellic acids represent lipophilic acids, it appeared reasonable to speculate that plasma protein (i.e. albumin)-binding of boswellic acids is responsible for the lack of efficacy in whole blood. Thus, we analyzed the albumin-binding ability of AKBA using a gel filtration method. As shown in Fig. 6, AKBA (50  $\mu$ M) extensively bound to 10 mg/ml BSA and to a minor extent when 1 mg/ml BSA was present. The unbound free AKBA was 0% and 30.9% for 10 and 1 mg/ml albumin, respectively.

Next, the effects of BSA on inhibition of 5-LO product synthesis by AKBA and KBA in isolated neutrophils was analyzed. The presence of 1 mg/ml BSA attenuated the potency of AKBA and KBA about 2- to 7-fold in neutrophils challenged by A23187 without (Fig. 7A) and with 20  $\mu$ M exogenous AA (Fig. 7B). When 10 mg/ml BSA was included, neither KBA nor AKBA significantly inhibited 5-LO product formation up to 30  $\mu$ M. Similarly, also the suppressive effect of H15® extract (30  $\mu$ g/ml) was completely reversed by 10 mg/ml BSA (not shown). We conclude that the strong BSA-binding capacity of boswellic acids



**Fig. 6.** Binding of boswellic acids to bovine serum albumin. BSA (0, 1 or 10 mg/ml, as indicated) was incubated with 50  $\mu$ M AKBA for 10 min at 37 °C. Separation of AKBA and albumin was carried out by gel filtration using a PD-10 column and AKBA was quantified by HPLC analysis. The concentration of AKBA (without BSA) versus elution volume was analysed. Based on this evaluation the amount of unbound AKBA was determined by the AUC ( $\mu$   $\pm$  3 $\sigma$ ) for incubation with 1 or 10 mg/ml albumin. Results shown are representative for at least 3 independent determinations.

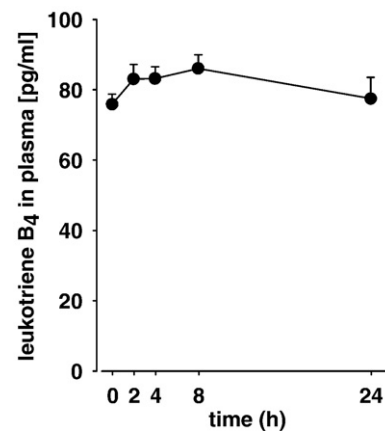


**Fig. 7.** Influence of bovine serum albumin on the efficacy of boswellic acids to inhibit 5-LO product formation in intact human neutrophils. Freshly isolated neutrophils ( $5 \times 10^6$ ) were resuspended in 1 ml PGC in the absence or presence of 1 or 10 mg/ml BSA and AKBA (left panel) or KBA (right panel), or vehicle (DMSO) were added at the indicated concentrations. After 10 min at 37 °C, cells were stimulated with 2.5  $\mu$ M A23187 (A) or with 2.5  $\mu$ M A23187 plus 20  $\mu$ M AA (B), incubated for another 10 min, and formation of 5-LO products (leukotriene B<sub>4</sub> and its all-trans isomers, 5(S),12(S)-DiHETE, and 5-H(P)ETE) was determined. 5-LO product synthesis in the absence of test compounds (100%, control) was  $68.1 \pm 6$  (A23187),  $40.8 \pm 3$  (A23187 + 1 mg/ml BSA),  $22.2 \pm 4$  (A23187 + 10 mg/ml BSA),  $276.8 \pm 40$  (A23187 + AA),  $78.3 \pm 7$  (A23187 + AA + 1 mg/ml BSA), and  $32.3 \pm 6$  (A23187 + AA + 10 mg/ml BSA) ng per  $10^6$  neutrophils. Values are given as mean  $\pm$  S.E.M.,  $n=3$ .

prevents inhibition of 5-LO product formation in isolated neutrophils and that this property is responsible for the failure of boswellic acids to inhibit 5-LO activity in whole blood.

### 3.5. Leukotriene B<sub>4</sub> levels in human subjects treated with frankincense extracts

In a phase 1 clinical trial the safety, tolerability and pharmacokinetics of a single oral dose application of *B. serrata* resin extract (PS0201Bo) in healthy male volunteers ( $n=12$ ) was investigated. Two capsules of PS0201Bo, each containing 400 mg of native *B. serrata* resin extract (total 800 mg PS0201Bo), were administered to healthy volunteers. Then, blood was collected as indicated in Fig. 8, plasma was prepared, and leukotriene B<sub>4</sub> was analyzed by ELISA. The plasma levels of leukotriene B<sub>4</sub> were not impaired within 24 h of frankincense administration, instead a weak but statistically not significant increase was observed 2 to 8 h after intake of the extracts, correlating with the plasma concentrations of AKBA and KBA that peaked after approx. 3 h, and half maximal concentrations of KBA were determined after 6–8 h (not shown).



**Fig. 8.** Effects of single dose orally administered frankincense preparation on leukotriene B<sub>4</sub> plasma levels. Healthy male volunteers received two capsules containing frankincense extract (PS 0201Bo) as single dose (800 mg) application. Venous blood was taken at the indicated time points, plasma was prepared and leukotriene B<sub>4</sub> in the plasma was determined by ELISA. Data are shown as mean  $\pm$  S.E.M.,  $n=12$  volunteers.

#### 4. Discussion

Here we provide a detailed analysis of the interference of boswellic acids with 5-LO. On one hand, we investigated mechanistic aspects of 5-LO inhibition by the major boswellic acids from frankincense applying cell-free and cell-based assays, taking into account the complex regulation of 5-LO activity. On the other hand, we evaluated the pharmacological relevance of 5-LO as target of boswellic acid using physiologically relevant test systems. In agreement with previous studies (Safayhi et al., 1997, 1995; Sailer et al., 1996; Werz et al., 1997), we find marked inhibition of human 5-LO by 11-keto-boswellic acids in cell-free and cell-based assays, supporting that boswellic acids are direct inhibitors of 5-LO. However, boswellic acids failed in test systems that reflect physiological conditions *in vivo* such as inclusion of albumin in neutrophil incubations or in human whole blood assays. Notably, leukotriene B<sub>4</sub> levels in human subjects treated with frankincense extracts are also not suppressed *in vivo*. We conclude that despite the unequivocal interference of boswellic acids with 5-LO *in vitro*, the failure of boswellic acids to reduce leukotriene formation in physiologically relevant bioassays and *in vivo* raises doubts about the pharmacological relevance of 5-LO inhibition by boswellic acids as underlying mode of action of the anti-inflammatory properties of frankincense.

5-LO activity is modulated by several co-factors (e.g. lipid hydroperoxides, phosphorylation events (Rådmark et al., 2007; Werz and Steinhilber, 2005)) that often affect the potency of pharmacological 5-LO inhibitors (Fischer et al., 2004, 2003; Werz and Steinhilber, 2005; Werz et al., 1998). 5-LO inhibitors are categorized as redox-type inhibitors that reduce the active site iron or scavenge stimulatory oxidants, iron ligand-type inhibitors that chelate the iron, and nonredox-type 5-LO inhibitors that may compete with AA or activating lipid hydroperoxides at distinct sites of 5-LO (Werz and Steinhilber, 2005). AKBA lacks antioxidant and iron-chelating properties and was proposed to act directly on 5-LO at a regulatory and selective site for pentacyclic triterpenes that is different from the AA-binding site (Safayhi et al., 1995; Sailer et al., 1998). Binding of 4-azido-5-<sup>125</sup>I-iodo-salicyloyl-β-alanyl-11-keto-boswellic acid to 5-LO was supported by Ca<sup>2+</sup> (>500 nM) but reduced by AA (10–50 μM), suggesting that AKBA binds in an AA-competitive and Ca<sup>2+</sup>-depending manner (Sailer et al., 1998). However, we found that AKBA and KBA were significantly more efficient at high AA concentrations, and also in intact neutrophils, supplementation of excess of AA (20 μM) rather improved the efficacy, excluding an AA-competitive mode of action. Moreover, Ca<sup>2+</sup> did not enhance the potencies against 5-LO activity but even slightly impaired it. Possibly, binding to 5-LO and inhibition of 5-LO catalytic activity is conferred by boswellic acids at different sites of the enzyme. In fact, also classical nonredox-type 5-LO inhibitors (e.g. ZM230487 and L-739,010) may act at two different (AA-binding) clefts on 5-LO with distinct affinities (Werz et al., 1998). Finally, in contrast to classical nonredox-type 5-LO inhibitors (Fischer et al., 2004; Werz et al., 1998), AKBA does not require glutathione peroxidase activity for efficient 5-LO inhibition which is also unaffected by 5-LO phosphorylation (Fischer et al., 2003). Nevertheless, AKBA was less active in the presence of phosphatidylcholine and when crude 5-LO in supernatants was analysed, which is not readily understood but at least indicates an interference with the regulatory phosphatidylcholine-binding C2-like domain or other putative regulatory sites (Rådmark et al., 2007).

Whereas cell-free assays are suitable to define molecular drug-target interactions, cell-based test systems represent the first step to estimate the efficacy of a given inhibitor towards the target *in vivo*. However, inhibition of 5-LO by AKBA and KBA (IC<sub>50</sub> = 1.5 and 2.8 μM) was originally demonstrated using neutrophils from rats (Safayhi et al., 1992, 1995; Sailer et al., 1996). Our experiments using neutrophils from humans confirm previous findings, but provided higher IC<sub>50</sub> values (3.2 and 8.8 μM for AKBA and KBA, respectively).

Species-related differences in the susceptibility of 5-LO towards boswellic acids are likely responsible, supported by the finding that AKBA was even less potent in neutrophils from guinea pigs (Wildfeuer et al., 1998). Also by using human cell lines MM6 and HL-60 higher IC<sub>50</sub> values for AKBA (12 and 15 μM, respectively, Werz et al., 1997) were determined.

Assays based on the use of isolated cells often neglect important parameters (i.e. albumin-binding, regulatory plasma components) that influence the bioactivity of test compounds *in vivo* and inclusion of some of these variables might better resemble the *in vivo* situation and anticipate *in vivo* outcomes. For example, LPS- and fMLP-evoked leukotriene formation in whole blood is considered to closely mimic pathophysiological conditions in the body (Surette et al., 1993), and is thus considered an appropriate test system to estimate the efficacy of 5-LO inhibitors *in vivo*. In our experiments using human activated neutrophils in the presence of albumin or using whole blood, boswellic acids and frankincense extracts completely failed to inhibit 5-LO. We hypothesize that the high albumin concentration in the whole blood abolishes 5-LO inhibition by AKBA and KBA. Thus, inclusion of BSA abolished inhibition of 5-LO product synthesis in neutrophil incubations and AKBA strongly bound to BSA. Albumin is abundant in plasma (30–40 mg/ml), and lipophilic acids (e.g. acidic drugs such as NSAIDs, warfarin, tolbutamide) are known to be substantially bound to serum albumin (Warner et al., 2006). Such (unspecific) protein-binding often impairs the pharmacological activity of the respective drug, and also for leukotriene synthesis inhibitors (e.g. MK-886) a strong loss of potency (approx. 500 fold) is evident in whole blood, apparently due to albumin-binding (Werz, 2002). Also boswellic acids are lipophilic acids explaining the tight binding to albumin and the loss of potency in the presence of albumin. Conclusively, albumin-binding may account for the failure of AKBA and KBA to inhibit 5-LO product synthesis in whole blood assays.

Leukotrienes play roles in inflammatory diseases and associations are established for asthma and allergic rhinitis, and anti-leukotrienes are used to treat these disorders (Peters-Golden and Henderson, 2007). However, increasing evidence from clinical trials and 5-LO-deficient mice exclude functions of leukotrienes in arthritis and inflammatory bowel diseases (Peters-Golden and Henderson, 2007; Werz and Steinhilber, 2006). Because frankincense is most efficient in the treatment of Crohn disease, colitis, and osteoarthritis (Ammon, 2006), interference with leukotriene synthesis is rather unlikely as responsible mechanism. Moreover, the pharmacological profile of potent well-established 5-LO inhibitors or leukotriene receptor antagonists *in vivo* clearly differs from that of frankincense preparations. Whether the interference of boswellic acids with alternative targets (e.g., IκB kinases or cyclooxygenase-1) accounts for the anti-inflammatory effectiveness of frankincense remains to be shown.

Frankincense extracts usually contain much more β-BA and α-BA than 11-keto-boswellic acids (Sterk et al., 2004) that also correlates to the significant lower plasma levels of 11-keto-boswellic acids after oral administration of frankincense preparations (Buchele and Simmet, 2003). In clinical trials, the maximal steady-state plasma levels of AKBA and KBA after oral application of 2.8 to 3.2 g per day frankincense extract were in the range of 0.04–0.1 and 0.3–0.33 μM, respectively (Buchele, and Simmet, 2003). In view of these fairly low plasma levels and in consideration of the failure of boswellic acids or frankincense extracts to inhibit 5-LO in whole blood, suppression of leukotriene synthesis *in vivo* by administered frankincense preparations is questionable. Finally, upon single administration of 800 mg frankincense extract to human volunteers the leukotriene B<sub>4</sub> plasma levels were not reduced. Similarly, Wildfeuer et al. (1998) failed to demonstrate suppression of leukotriene B<sub>4</sub> and C<sub>4</sub> *ex-vivo* in A23187-challenged neutrophils from guinea pigs treated (i.p.) with 20 mg/kg acetyl-boswellic acids.

In summary, boswellic acids are direct inhibitors of 5-LO with a mechanistically unique mode of action, influenced by 5-LO-



modulating co-factors and by experimental assay settings. 5-LO products play pivotal roles in certain inflammatory diseases, but the beneficial effects of frankincense preparations are unlikely based on the interference of 11-keto-boswellic acids with 5-LO. In particular, the fairly low plasma levels of 11-keto-boswellic acids obtained after oral administration of frankincense accompanied by the lack of reduction of leukotriene B<sub>4</sub> plasma levels, and the failure of 11-keto boswellic acids to inhibit 5-LO product formation in whole blood raises doubts regarding the interference of boswellic acids with 5-LO *in vivo* and questions its pharmacological relevance.

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