BIOCHEMICAL PHARMACOLOGY 75 (2008) 503–513

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Identification and functional analysis of cyclooxygenase-1 as a molecular target of boswellic acids

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ARTICLE INFO

Article history:
Received 10 August 2007
Accepted 10 September 2007

Keywords:
Boswellic acids
Cyclooxygenase
Lipoxygenase
Inflammation
Platelets
Prostaglandin

ABSTRACT

Boswellic acids (BAs) are assumed as the anti-inflammatory principles of *Boswellia* species. Initially, it was found that BAs inhibit leukotriene biosynthesis and 5-lipoxygenase (EC number 1.13.11.34), whereas suppression of prostaglandin formation and inhibition of cyclooxygenases (COX, EC number 1.14.99.1) has been excluded. Recently, we demonstrated that BAs also interfere with platelet-type 12-lipoxygenase. Here, we show that BAs, preferably 3-*O*-acetyl-11-keto-β-BA (AKBA), concentration-dependently inhibit COX-1 product formation in intact human platelets (IC_{50} = 6 μM) as well as the activity of isolated COX-1 enzyme in cell-free assays (IC_{50} = 32 μM). The inhibitory effect of AKBA is reversible, and increased levels of arachidonic acid (AA) as substrate for COX-1 impair the efficacy. COX-1 in platelet lysates or isolated COX-1 selectively bound to an affinity matrix composed of immobilized BAs linked via glutaric acid to sepharose and this binding was reversed by ibuprofen or AA. Automated molecular docking of BAs into X-ray structures of COX-1 yielded positive Chemscore values for BAs, indicating favorable binding to the active site of the enzyme. In contrast, COX-2 was less efficiently inhibited by BAs as compared to COX-1, and pull-down experiments as well as docking studies exclude strong affinities of BAs towards COX-2. In conclusion, BAs, in particular AKBA, directly interfere with COX-1 and may mediate their anti-inflammatory actions not only by suppression of lipoxygenases, but also by inhibiting cyclooxygenases, preferentially COX-1.

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Abbreviations: AB, antibodies; Aβ-BA, 3-*O*-acetyl-boswellic acid; AKBA, 3-*O*-acetyl-11-keto-boswellic acid; BA, boswellic acid; β-BA, β-boswellic acid; COX, cyclooxygenase; KBA, 11-keto-boswellic acid; LO, lipoxygenase; MAPK, mitogen-activated protein kinase; PG buffer, PBS plus 1 mg ml^{-1} glucose; PGC buffer, PBS containing 1 mg ml^{-1} glucose and 1 mM $CaCl_2$; PMNL, polymorphonuclear leukocytes; RMSD, root mean square deviation; RT, room temperature; SDS-b, 2 × sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample loading buffer.

0006-2952/$ – see front matter
doi:10.1016/j.bcp.2007.09.010

1. Introduction

Extracts of the gum resins of *Boswellia* species are traditionally applied in folk medicine to treat various chronic inflammatory diseases such as rheumatoid arthritis, inflammatory bowel diseases, and asthma, and experimental data from animal models and clinical studies on humans confirmed an anti-inflammatory potential of *Boswellia* species preparations (for review, see ref. [1]). The pentacyclic triterpenes boswellic acids (BAs) (Fig. 1) possess diverse pharmacological properties including antiproliferative, pro-apoptotic and pro-differentiating, and anti-inflammatory effects, and are assumed as the active principles of *Boswellia* species extracts (for review, see ref. [2]).

A number of effects of BAs on signalling pathways relevant for inflammation such as the NFκB route [3] and the MAPK pathway [4], as well as on effector molecules including 5-lipoxygenase (5-LO, EC number 1.13.11.34) [5], platelet-type 12-lipoxygenase (p12-LO) [6] and human leukocyte elastase (HLE) [7] were described. Accordingly, it is speculated that BAs may exert their anti-inflammatory effect mainly by inhibiting the release of proinflammatory LO products from leukocytes and platelets [5,6] and by inhibition of NF-κB and subsequent down-regulation of TNF-alpha expression in activated monocytes [3].

Eicosanoids, generated from arachidonic acid (AA) via the 5-LO and cyclooxygenase (COX, EC number 1.14.99.1)-1/2 pathways, function as potent mediators of inflammatory processes [8]. These bioactive lipid mediators (i.e. leukotrienes and prostaglandins (PGs), respectively) act via specific G protein-coupled receptors, and inhibitors of 5-LO and COX-1/2 as well as eicosanoid receptor antagonists comprise an important pharmacological tool for the treatment of inflammatory diseases [8]. In particular, the so-called NSAIDs that block COX-1/2, and compounds that selectively inhibit COX-2, are frequently applied in therapy of pain, fever and rheumatoid arthritis [9]. The two COX isoenzymes share ~60% sequence identity and perform the first committed steps in the PG biosynthetic pathway by dioxygenation of AA to PGG_2 as well as the subsequent reduction of PGG_2 to PGH_2 [10]. Whereas COX-1 is a constitutively expressed enzyme in numerous cell types and thought to provide PGs mainly for physiological functions governing cellular homeostasis, COX-2 is an inducible variant in inflammatory cells and inflamed tissue, mainly producing PGs relevant for inflammation, fever and pain [9]. However, a number of recent clinical studies raised doubts about the usefulness of highly selective COX-2 inhibitors, particularly due to their small but significant increased risk for cardiovascular events [11].

Initial studies aiming to reveal the mode of action of BAs identified 5-LO as target. Thus, BAs suppressed the formation of LTs in activated intact leukocytes [5,12,13], blocked 5-LO activity in cell-free assays [14,15] and a photoaffinity analogue of AKBA (able to inhibit 5-LO activity) specifically labeled human 5-LO [16]. Interestingly, one of the initial reports addressing the actions of BAs on eicosanoid biosynthesis suggested that BAs are specific for 5-LO without affecting other LOs (i.e. human p12-LO) or COX-1 in concentrations up to 400 μM [5,17]. We recently demonstrated that AKBA inhibits p12-LO and directly binds to this enzyme [6]. Here, we show for the first time that BAs are able to bind and to inhibit COX-1.

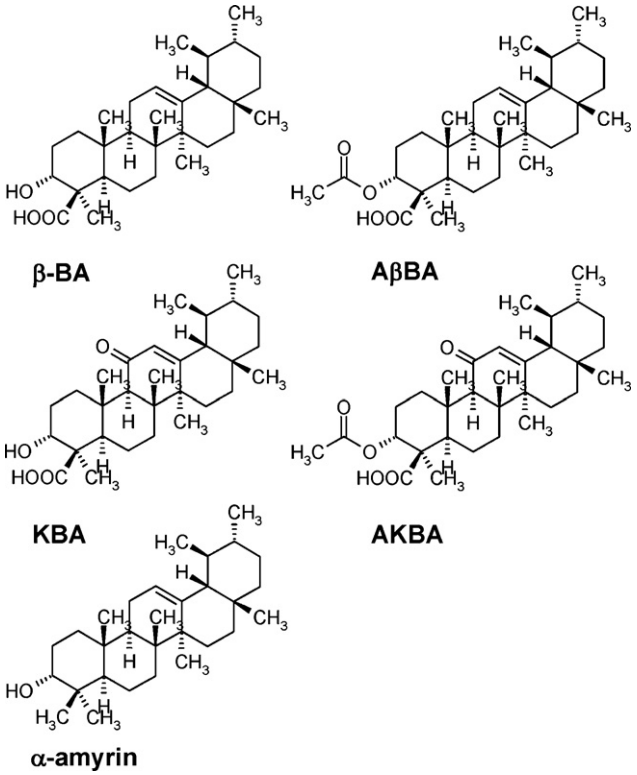

Fig. 1 – Chemical structures of BAs and α-amyrin.

2. Materials and methods

2.1. Reagents

BAs were prepared as described previously [18]. Calcitriol was a kind gift from Schering (Berlin, Germany). Anti-mouse-IgG-6-keto $PGF1_\alpha$-antibody was kindly provided by Dr. T. Dingermann (Frankfurt, Germany). [^{3}H]AA was from Biotrend (Cologne, Germany). COX-1 (ovine), COX-2 (human recombinant), 6-keto-$PGF_{1\alpha}$, and antibodies against COX-1 were from Cayman Chemical (Ann Arbor, MI, USA); antibodies against COX-2 were from Biomol Intern. (Hamburg, Germany); α-amyrin was from Extrasynthèse (Genay, France); EAH-Sepharose 4B, GE Healthcare Bio-Sciences (Freiburg, Germany); cinnamyl-3,4-dihydroxy-α-cyanocinnamate (CDC), Biomol (Plymouth Meeting, PA); AA, lipopolysaccharide (LPS), thrombin and other chemicals were obtained from Sigma (Deisenhofen, Germany).

2.2. Cells

Platelets were freshly isolated from human venous blood of healthy adult donors (Blood Center, University Hospital, Tuebingen, Germany) that did not take any medication for at least 7 days, as described [19]. Briefly, platelet-rich-plasma (PRP) was obtained after centrifugation of freshly drawn whole blood (K-EDTA 1.6 mg ml^{-1} blood) at 150 × *g*, RT for 15 min. Washed platelets were finally resuspended in PBS pH 7.4 and 1 mg ml^{-1} glucose (PG buffer) or in PBS pH 7.4 and 1 mg ml^{-1}

glucose plus 1 mM $CaCl_2$ (PGC buffer). For incubations with solubilized compounds, ethanol or DMSO was used as vehicle, never exceeding 1% (v/v). The viability of platelets in response to any agent (BAs, thrombin, or Ca^{2+} ionophore A23187) was analyzed by determination of the particle distribution pattern using a Sysmex Cell Counter (Norderstedt, Germany) as described [19].

The human monocytic cell line Mono Mac (MM) 6 cells were maintained as described elsewhere [20]. These cells possess characteristics of mature monocytes [21] and express substantial amounts of COX-2 after differentiation with calcitriol and subsequent incubation with LPS [20]. To exclude toxic effects of BAs during preincubation periods, cell viability was analyzed by light microscopy and trypan blue exclusion. Incubation with 100 μM of any of the BAs at 37 °C for up to 30 min caused no significant change in MM6 cell viability.

2.3. Determination of release of [^{3}H]-labeled arachidonic acid from intact platelets

Platelet rich plasma was labelled with 19.2 nM [^{3}H]AA (1 μCi ml^{-1}, specific activity 200 Ci $mmol^{-1}$) for 2 h at 37 °C. Then, cells were washed twice with PBS pH 5.9 plus 1 mM $MgCl_2$, 11.5 mM $NaHCO_3$, 1 g l^{-1} glucose, and 1 mg ml^{-1} fatty acid-free BSA. Preparation of platelets at pH 5.9 is thought to minimize temperature-induced activation. Platelets (10^8 ml^{-1}) were finally resuspended in PGC buffer, pH 7.4, and subsequently stimulated with thrombin (2 U ml^{-1}), β-BA or AKBA (30 μM, each) for 5 min at 37 °C and then put on ice for 10 min, followed by centrifugation (5,000×*g*, 15 min). Aliquots (300 μl) of the supernatants were measured (Micro Beta Trilux, Perkin-Elmer) to detect the amounts of [^{3}H]-labeled AA released into the medium.

In order to ascertain that the radioactivity released is AA and not an AA metabolite, we directly analyzed the AA released from unlabelled platelets after separation by solid phase extraction, coupling to dimethoxyaniline hydrochloride (DMA-HCl) in presence of *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide (EDC) in methanol and the derivatized AA was analyzed by RP-HPLC at a wave length of 272 nm [22].

2.4. Determination of COX-1 product formation in washed platelets

Freshly isolated platelets (10^8 ml^{-1} PG buffer) were supplemented with 1 mM $CaCl_2$. Since BAs act differentially on p12-LO (11-methylene-BAs stimulate, 11-keto-BAs inhibit) and thus, to avoid differential conversion of AA by p12-LO, the selective 12-LO inhibitor CDC (10 μM) [23], commonly used to block 12-LO activity in studies of platelet functions [6,24], was included in all incubations to assure comparable AA levels for conversion by COX-1. Platelets were preincubated with the indicated agents for 5 min at RT. After addition of thrombin (2 U ml^{-1}), A23718 (2.5 μM) or AA (5 μM) and further incubation for 5 min at 37 °C, the COX-1 product 12(*S*)-hydroxy-5-*cis*-8,10-*trans*-heptadecatrienoic acid (12-HHT) was extracted and then analyzed by HPLC as described [20,25]. COX-1 product formation is expressed as ng of 12-HHT per 10^8 cells.

2.5. Determination of COX-2 product formation in intact MM6 cells

The release of 6-keto-$PGF_{1\alpha}$ from LPS-stimulated MM6 cells expressing COX-2 was assessed by ELISA as described [20]. In brief, MM6 cells were grown with 50 nM calcitriol for 96 h, LPS (100 ng ml^{-1}) was added and after 6 h, cells were harvested, resuspended in PGC buffer (3×10^6 cells ml^{-1}) and preincubated with the test compounds at the indicated concentrations for 15 min at 37 °C, and then stimulated with AA (30 μM) for another 15 min at 37 °C. The amount of 6-keto $PGF_{1\alpha}$ released was determined by ELISA using a monoclonal antibody against 6-keto $PGF_{1\alpha}$. The monoclonal antibody (0.2 μg in 200 μl) was coated to microtiter plates via a goat anti-mouse-IgG antibody. 6-Keto $PGF_{1\alpha}$ (15 μg) was linked to bacterial β-galactosidase (0.5 mg, Calbiochem) and the enzyme activity bound to the antibody was determined in an ELISA reader (SynergyHT, BioTEK) at OD550 nm (reference wavelength: 630 nm) using chlorophenol-red-β-D-galactopyranoside (Roche Diagnostic GmbH) as substrate.

2.6. Activity assays of isolated COX-1 and -2

Inhibition of the activities of isolated ovine COX-1 and human COX-2 by BAs was performed as described [26,27]. Though the purified COX-1 is not of human origin, ovine COX-1 is generally used for inhibitor studies when examining the effectiveness of compounds on the activity of isolated COX-1 enzyme [26,37]. Briefly, purified COX-1 (ovine, 50 units) or COX-2 (human recombinant, 20 units) were diluted in 1 ml reaction mixture containing 100 mM Tris buffer pH 8, 5 mM glutathione (GSH), 5 μM haemoglobin, and 100 μM EDTA at 4 °C and preincubated with the test compounds for 5 min. Samples were pre-warmed for 60 s at 37 °C and AA (5 μM) was added to start the reaction. After 5 min at 37 °C, 12-HHT was extracted and then analyzed by HPLC as described for intact platelets (see above). COX-1/2 product formation is expressed as ng of 12-HHT per mg min^{-1} COX enzyme.

2.7. Immobilization of boswellic acids and protein pull-down assays

For immobilization of KBA or β-BA at EAH Sepharose 4B beads, the free 3-OH group of KBA was used (manuscript in preparation: Kather, N., Tausch, L., Poeckel, D., Werz, O., Herdtweck, E. and Jauch, J., 2007). In brief, KBA or β-BA were treated with glutaric anhydride to form the half-ester Glut-KBA or Glut-β-BA (analyzed by ^{1}H and ^{13}C NMR and by MS) which were immobilized at EAH Sepharose 4B by standard amide coupling procedures. The carboxylic acid of the BA-core was unlikely to react under standard conditions due to steric crowding. The success of the coupling reaction was determined by two methods: (a) Glut-KBA was used in defined excess (2 μmol of Glut-KBA per 1 μmol NH_2-groups of the EAH Sepharose 4B). After the coupling reaction, the hypothetical excess of Glut-KBA (1 μmol) could be indeed recovered. (b) Treatment of Glut-KBA with KOH in *iso*-propanol under reflux for approximately 3 h cleaved the ester bond and gave KBA, analyzed by thin layer chromatography.

For protein fishing experiments, 10^9 platelets or 3×10^7 MM6 cells were lysed in 1 ml lysis buffer (50 mM HEPES pH 7.4, 200 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2 mM PMSF, 2 μg ml^{-1} leupeptin, 120 μg ml^{-1} soybean trypsin inhibitor). After sonication (3 × 8 s) and centrifugation for 10 min at 12,000 × g, 500 μl of the sepharose slurries (50%, v/v) were added to supernatants and incubated at 4 °C over night under continuous rotation. For pull-down of purified COX-1, 10 units of the purified enzyme were diluted into 500 μl of lysis buffer containing 0.1 mg ml^{-1} BSA (as blocking agent), and 200 μl of sepharose slurry was added. The sepharose beads were washed 3 times with binding buffer (HEPES pH 7.4, 200 mM NaCl, 1 mM EDTA) and precipitated proteins were finally separated and denatured by addition of 2 × sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer (SDS-b; 20 mM Tris/HCl, pH 8, 2 mM EDTA, 5% SDS (w/v), 10% β-mercaptoethanol). After boiling (95 °C, 6 min), sepharose beads were removed by centrifugation and proteins in the supernatant were analyzed by SDS-PAGE with subsequent detection of proteins by WB, Coomassie or silver staining, respectively, as described [6].

2.8. *In-gel digestion and nanoflow liquid chromatography tandem MS (nano-LC–ESI-MS/MS)*

In-gel protein digestions were carried out overnight with trypsin (porcine, sequencing grade, modified; Promega, Mannheim, Germany) at 37 °C. Reversed-phase nano-LC–MS/MS was performed as described previously [28]. In brief, the tryptic peptide mixtures were autosampled in 0.1% aqueous trifluoroacetic acid, and desalted on a PepMap C18 trapping cartridge (LC Packings). The trapped peptides were eluted and separated on a PepMap C18 column (75 μm i.d. × 15 cm; LC Packings) using a linear gradient of acetonitrile in 0.1% (v/v) formic acid, and ionized by an applied voltage of 2200 kV to the emitter. The mass spectrometer was operated in data-dependent acquisition mode to automatically switch between MS and MS/MS. Survey MS spectra were acquired for 1 s, and the three most intense ions (doubly or triply charged) were isolated, and sequentially fragmented for 1.5 s by low-energy collision-induced dissociation. All MS and MS/MS spectra were acquired with the Q2-pulsing function switched on, and optimized for enhanced transmission of ions in the MS (*m*/*z* 400–1000) and MS/MS (*m*/*z* 75–1300) mass ranges. Proteins were identified by correlating the data from the MS/MS spectra with the NCBI.nr-protein sequence database (version 20060511, taxonomy Homo sapiens) using the MOWSE-algorithm as implemented in the search engine MASCOT (Matrix Science Ltd. London, UK).

2.9. *Automated docking*

Automated molecular docking of BAs into X-ray structures of COX-1 and COX-2 was performed using GOLD 3.1.1, which relies on a genetic algorithm for structure optimization [29]. We used the available crystal structure of COX-1, PDB-code 1Q4G at 2.0 Å resolution, with bound inhibitor 2-(1,1′-biphenyl-4-yl)propanoic acid (BFL), a defluorinated flurbiprofen analog [30]. For COX-2, we selected PDB entry 6COX at 2.8 Å resolution, complexed with the selective inhibitor SC-558 [31]. Hydrogens were added to the proteins, and energy minimized using the AMBER99 force field, within the software MOE 2006.08 (Chemical Computing Group, Montreal, Canada). For the co-crystallized inhibitors hydrogen atoms were added, and energy minimization was performed using the MMFF94x force field [32]. Starting conformations for the 3D structures of the four BAs were calculated with MOE using the MMFF94x force field. GOLD parameter settings for the genetic algorithm were: number of operations = 10,000, population size = 100, selection pressure = 1.1, number of islands = 1, niche size = 2, migrate = 0, mutate = 100, crossover = 100. A 10 Å radius around the bound inhibitor in the active site defined the binding pocket. The Chemscore function [33] was used for scoring the predicted binding poses. Greater positive score values indicate more favorable protein–ligand complexes, negative values indicate unfavorable binding modes (non-binding). Each docking run was repeated 10 times. The same method was used for re-docking of the co-crystallized inhibitors. Root mean square deviation (RMSD) values between the PDB X-ray structures and the docking solutions were computed, and a mean value with standard deviation was calculated. PyMOL was used for visualization of docking poses [34].

2.10. *Statistics*

Statistical evaluation of the data was performed by one-way ANOVAs for independent or correlated samples followed by Tukey HSD post hoc tests. A p value of <0.05 (*) or <0.01 (**) was considered significant. IC_{50} values for compounds examined on inhibition of COX-1/2 product formation are approximations determined by graphical analysis (linear interpolation between the points between 50% activity).

3. Results

3.1. *BAs suppress COX-1 product formation in washed human platelets*

Previously, we found that various BAs induce the release of AA from human washed platelets, but only 11-methylene-BAs caused transformation of AA to 12-H(P)ETE, whereas 11-keto-BAs failed in this respect [6]. Besides p12-LO, COX-1 is a major enzyme in platelets converting AA into oxidised metabolites including 12-HHT [35,36]. We determined the effects of an 11-methylene-BA (i.e. β-BA) and an 11-keto-BA (i.e. AKBA) on 12-HHT formation in platelets from endogenous AA (Table 1). Both, β-BA and AKBA (30 μM, each) caused AA release comparable to thrombin (2 U ml^{-1}). Accordingly, β-BA (and thrombin) induced formation of 12-HHT, but AKBA was hardly effective (Table 1), despite its ability to release AA as substrate for COX-1. This suggests that AKBA may have an inhibitory effect on COX-1.

In order to determine if BAs (in particular AKBA) may inhibit agonist-induced COX-1 product formation, human washed platelets were preincubated with BAs and stimulated with thrombin or Ca^{2+}-ionophore A23187 (circumventing receptor signalling). AKBA and KBA concentration-dependently inhibited COX-1 product synthesis in platelets stimulated with A23187 (Fig. 2A) or thrombin (Fig. 2B). For AKBA, the

Table 1 – Induction of AA release and 12-HHT formation in washed human platelets

Compound	AA release (cpm)	12-HHT formation (ng per 10^8 cells)
Vehicle	445 ± 173	3.5 ± 1.3
Thrombin (2 U ml^{-1})	1255 ± 180	165.4 ± 39.4
β-BA (30 μM)	921 ± 101	141.2 ± 5.1
AKBA (30 μM)	1045 ± 117	5.4 ± 1.9

For determination of AA release, platelets (10^8 ml^{-1} PGC buffer) were incubated with the indicated agents for 5 min at 37 °C, DMSO was used a vehicle. [^{3}H]AA released into the medium was measured as described. Data are given as cpm, mean + S.E., $n = 5$. For determination of 12-HHT formation, platelets (10^8 ml^{-1} PGC buffer) were incubated with the indicated agents for 5 min at 37 °C and 12-HHT formation was determined. Data are given as ng of 12-HHT formed by 10^8 platelets, mean + S.E. of $n = 4$.

IC_{50} values were determined at approximately 6 μM (stimulation with A23187) and 17 μM (stimulation with thrombin) and for KBA at approximately 14 and 55 μM, respectively. In contrast, the 11-methylene-BAs are less potent COX-1 inhibitors with IC_{50} values >100 μM, and for thrombin-activated platelets, 11-methylene-BAs even slightly enhanced 12-HHT formation (Fig. 2B). The structural BA analogue α-amyrin (100 μM, negative control) gave no significant inhibition (88.4 ± 4.6% of control), whereas the reference drugs aspirin (100 μM) and ibuprofen (30 μM) efficiently blocked thrombin-induced 12-HTT formation (3.1 ± 0.5% and 5.9 ± 1.8% of control, respectively).

To confirm that BAs inhibit COX-1 and to exclude suppressive effects of BAs on agonist-induced AA supply we next assessed the effects of BAs on 12-HHT formation in platelets that received exogenously added AA (5 μM). As shown in Fig. 2C, a concentration-dependent inhibition of 12-HHT formation was observed for all BAs with AKBA being most potent (IC_{50} value approximately 23 μM), whereas the IC_{50} values for all other BAs were >50 μM. The negative control α-amyrin was not active up to 100 μM (Fig. 2C), and aspirin (100 μM) or ibuprofen (30 μM) inhibited 12-HTT formation as expected (18 ± 9% and 23.8 ± 6.4% of control, respectively).

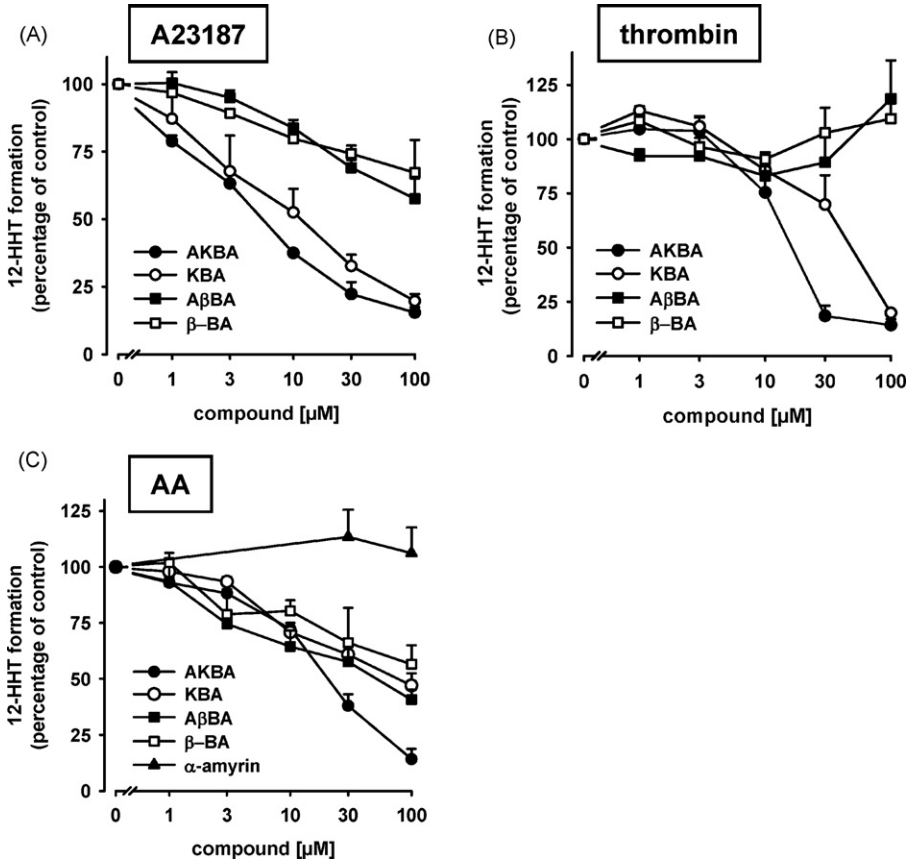

Fig. 2 – Inhibition of COX-1 activity by BAs in intact human platelets. Washed human platelets (10^8 ml^{-1} PGC buffer) were incubated with the indicated concentrations of the BAs or α-amyrin. After 5 min at RT: (A) 2.5 μM A23187, (B) 2 U ml^{-1} thrombin, or (C) 5 μM AA was added to induce COX-1 product formation. After another 5 min at 37 °C the reaction was terminated and 12-HHT was determined. Data are given as mean + S.E., $n = 4–6$. 12-HHT formation in the absence of test compounds (100%, control) was 104.6 ± 8.9 (ionophore), 165.4 ± 39.4 (thrombin), and 186.4 ± 17.6 (AA) ng per 10^8 platelets.

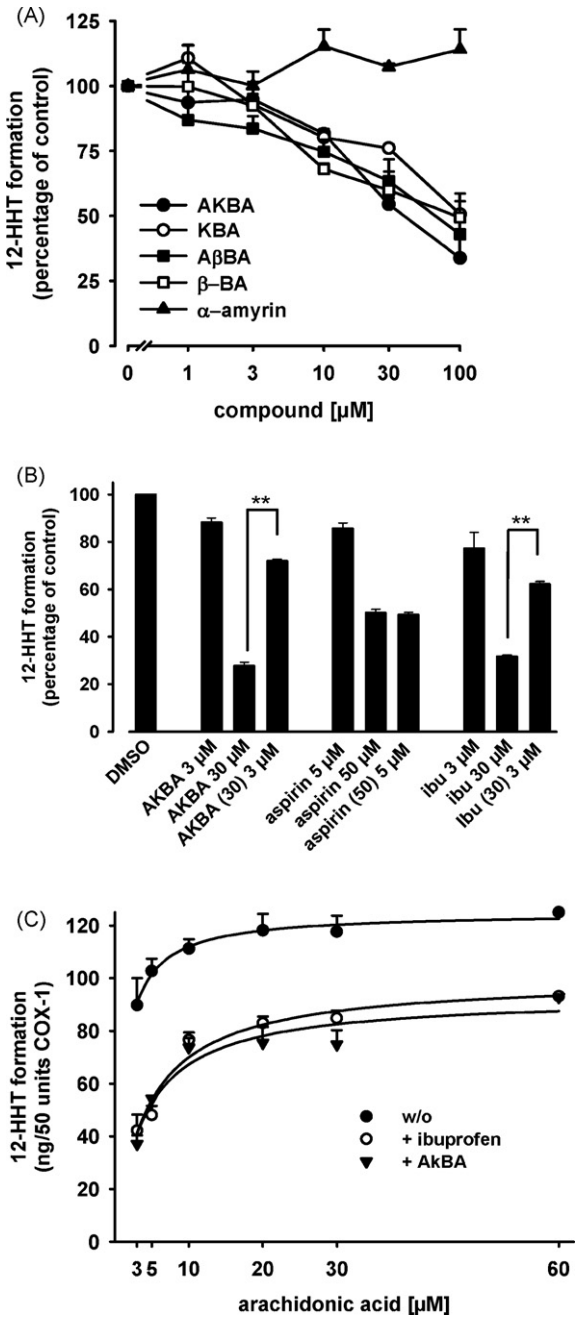

Fig. 3 – Inhibition of COX-1 activity by BAs in cell-free assays. (A) Concentration–response studies. Isolated COX-1 (50 units) was diluted in 1 ml reaction mixture (see Section 2) and preincubated with the BAs or α-amyrin for 5 min at 4 °C. Samples were then prewarmed for 60 s at 37 °C and 5 μM AA was added to start the reaction. After 5 min at 37 °C, 12-HHT was analyzed as described. (B) Reversibility of COX-1 inhibition. Isolated COX-1 (50 units) was incubated with or without 30 μM AKBA, 50 μM aspirin, 30 μM ibuprofen, or vehicle (DMSO) for 5 min at RT, each. Then, one aliquot of the samples was diluted with assay buffer 10-fold whereas the other one was not altered, and 5 μM AA was added to start the COX-1 reaction. For comparison, COX-1 was preincubated for 5 min at RT with 3 μM AKBA, 5 μM aspirin, or 3 μM ibuprofen and then 5 μM AA was added (no dilution). Then, all samples were incubated for 5 min at 37 °C and 12-HHT was analyzed. (C) Effects of variation of the AA concentration. Isolated COX-1 (50 units) was diluted in 1 ml reaction mixture, preincubated with the 30 μM AKBA or 20 μM ibuprofen for 5 min at 4 °C, the samples were prewarmed for 60 s at 37 °C, and the indicated concentrations of AA were added. After 5 min, 12-HHT was analyzed. All data are given as mean + S.E., $n = 4–5$. One-way ANOVA and Tukey HSD post hoc tests were performed; $^{**}p < 0.01$ vs. vehicle control (DMSO).

3.2. BAs suppress the activity of isolated COX-1

To determine if BAs directly interfere with COX-1 activity, isolated ovine COX-1 was incubated with 5 μM AA in the presence of GSH, leading to generation of 12-HHT as the major COX-1 product [27]. As shown in Fig. 3A, all BAs reduced the activity of COX-1. The IC_{50} value for AKBA was determined at approximately 32 μM being almost equipotent with ibuprofen (IC_{50} = 25 μM, not shown), and α-amyrin (up to 100 μM) failed to suppress 12-HHT synthesis (Fig. 3A).

Aspirin causes acetylation of Ser530 in COX-1 resulting in irreversible enzyme inhibition [38], whereas ibuprofen and other NSAIDs are reversible COX-1 inhibitors [39]. To investigate if AKBA blocks COX-1 in a reversible manner, COX-1 was incubated with or without 30 μM AKBA, 50 μM aspirin or 30 μM ibuprofen for 10 min at RT, each. Then, samples were splitted and one aliquot was diluted with assay buffer 10-fold, whereas the other one was not altered, and 5 μM AA was added to each aliquot to start the COX-1 reaction. As shown in Fig. 3B, dilution of COX-1 incubations with 50 μM aspirin to a final concentration of 5 μM caused no change in the magnitude of COX-1 inhibition, but dilution of incubations containing AKBA or ibuprofen resulted in a significant loss of inhibition. Hence, we conclude that AKBA (like ibuprofen but unlike aspirin) may act as a reversible inhibitor of COX-1.

To determine whether COX-1 inhibition by AKBA is affected by the AA substrate concentration, assays were performed at increasing amounts of AA in the absence or the presence of 30 μM AKBA or 20 μM ibuprofen. Potent inhibition of COX-1 was obvious for AKBA and ibuprofen at low (3 or 5 μM) substrate concentrations but modest inhibition was observed at high (60 μM) amounts of AA (Fig. 3C). Thus, AA reduces the efficacy of AKBA.

3.3. COX-1 binds to immobilized BAs

Recently, we showed that p12-LO binds to KBA immobilized at EAH-Sepharose beads [6]. The same ligand/protein-binding strategy was used to assess the binding of BAs to COX-1. KBA or β-BA were used as bait, covalently linked to EAH Sepharose 4B beads via a glutaric acid linker (KBA-Seph, β-BA-Seph), and EAH-Sepharose 4B beads without ligand (Seph) were used as negative control. Platelet lysates (as source for COX-1) were incubated with the sepharose beads, precipitated proteins were separated by SDS-PAGE and visualized by Coomassie- or silver-staining. Comparison of fished proteins using both sepharose beads revealed a protein band with an apparent mass of 72 kDa in samples of β-BA-Seph and KBA-Seph, but not in controls where sepharose beads without ligand (Seph)

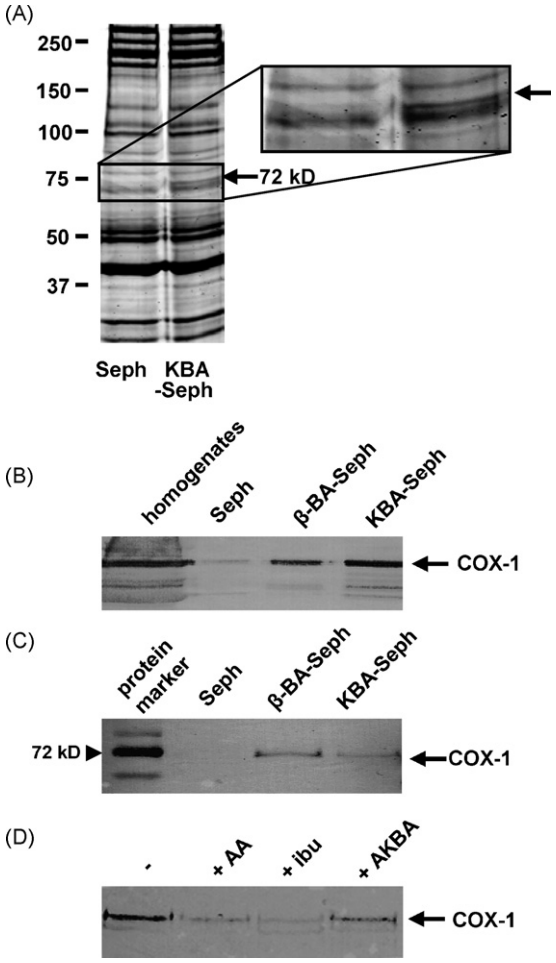

Fig. 4 – Pull-down of COX-1 by immobilized BAs. 12,000 × *g* supernatants of platelet lysates were incubated over night at 4 °C with either β-BA-Seph, KBA-Seph or with crude Seph, as indicated. Precipitates were intensively washed, solubilized by addition of SDS-b and separated by SDS-PAGE. (A) Proteins were visualized by silver-staining, bands of interest at 72 kDa were excised, in-gel digested and analyzed by LC–ESI-MS/MS. (B) Proteins were visualized by Western-blotting using specific antibodies against COX-1. An aliquot of platelet 12,000 × *g* supernatant was used as a positive control. (C) Isolated COX-1 (50 ng) was incubated with β-BA-Seph, KBA-Seph, or Seph, in the presence of BSA (0.1 mg ml^{-1}). (D) Isolated COX-1 (50 ng) was incubated as above with or without AA (50 μM), ibuprofen (50 μM) or AKBA (100 μM), as indicated. Precipitates were intensively washed, solubilized and separated by SDS-PAGE as described above. COX-1 was visualized by Western blotting; correct loading was controlled by analysis of albumin (that unspecifically bound to the beads) using Ponceau S staining (not shown). Similar results were obtained in three additional experiments.

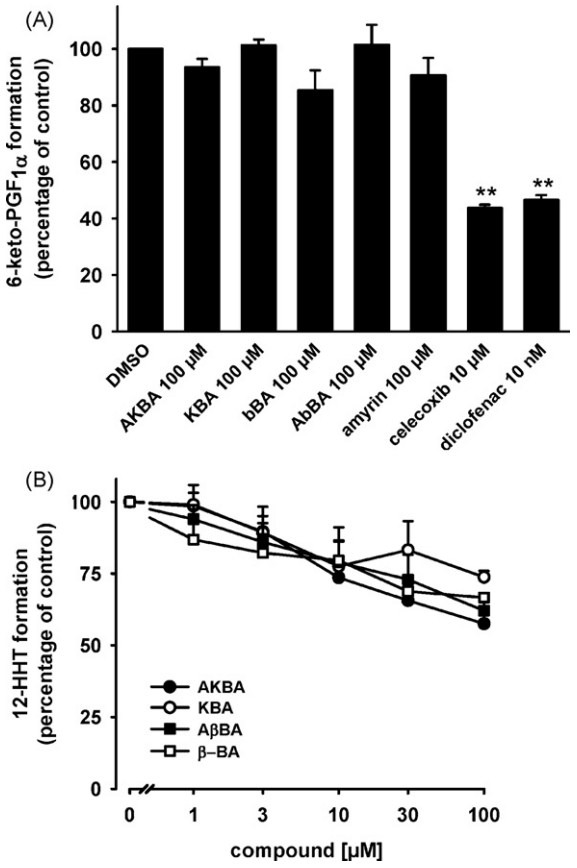

Fig. 5 – Inhibition of COX-2. (A) Inhibition of COX-2 in intact MM6 cells. MM6 cells were grown in presence of calcitriol (50 nM) for 96 h, and LPS (100 ng ml^{-1}) was added 6 h prior cell harvest. Cells (3 × 10^6 ml^{-1} PGC) were first preincubated with BAs, celecoxib (10 μM), diclofenac (10 nM), or vehicle (DMSO) for 15 min at 37 °C and then AA (30 μM) was added to start the COX-2 product formation. After 15 min at 37 °C the formed amounts of 6-keto $PGF_{1\alpha}$ were determined by ELISA as described. Results are given as mean + S.E., $n = 3$. Formation of 6-keto $PGF_{1\alpha}$ in the absence of test compounds (100%, control) was 2.1 ± 0.2 ng per 10^6 MM6 cells. One-way ANOVA and Tukey HSD post hoc tests were performed; $^{**}p < 0.01$ vs. vehicle control (DMSO). (B) Inhibition of COX-2 in cell-free assays. Isolated human recombinant COX-2 (20 units) was diluted in 1 ml reaction mixture and preincubated at 4 °C with the test compounds for 5 min. Samples were then prewarmed for 60 s at 37 °C and 5 μM AA was added. After 5 min, 12-HHT formation was determined by HPLC. Results are given as mean + S.E., $n = 4$.

were utilized (Fig. 4A). In-gel trypsin digestion and subsequent nano-LC–ESI-MS/MS analysis revealed that the 72 kDa protein is human COX-1, confirmed by WB using specific AB against COX-1 (Fig. 4B). To exclude an indirect precipitation of COX-1 via any linker molecule present in platelet lysates (that could actually be the binding partner of BAs), isolated ovine COX-1 (50 ng) in the absence of platelet proteins was incubated with Seph, KBA-Seph, and β-BA-Seph in the presence of BSA (0.1 mg ml^{-1}, as blocking agent). Again, KBA-Seph, and β-BA-Seph precipitated COX-1 but not so Seph without ligand (Fig. 4C).

Using the pull-down strategy described above, the interaction between COX-1 and KBA-Seph was further character-

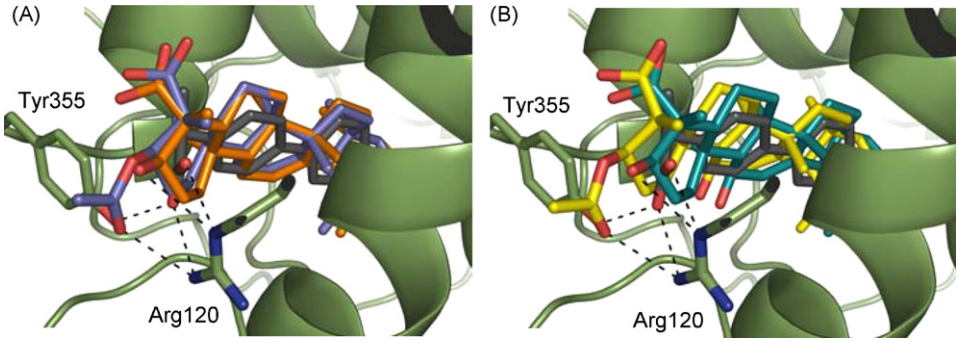

Fig. 6 – Automated molecular docking of BAs into X-ray structures of COX-1. (A and B) Schematic of the active site of COX-1 (1Q4G) with the co-crystallized inhibitor 2-(1,1′-biphenyl-4-yl)propanoic acid (BFL) displayed in grey. Possible hydrogen bonds of the BAs formed with R120 and Y355 are indicated. (A) β-BA (orange) and Aβ-BA (lilac) are shown. (B) KBA (green) and AKBA (yellow) are shown in the same orientation as BFL (grey). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

ized. Co-incubation with AA (50 μM) or ibuprofen (50 μM) strongly reduced the amounts of COX-1 that bound to KBA-Seph, indicating that KBA-Seph may interact at the same AA-binding site that is bound by ibuprofen (Fig. 4D). Furthermore, AKBA (100 μM) competed with KBA-Seph for binding to COX-1.

3.4. *BAs moderately interfere with COX-2*

Next, the effects of BAs on COX-2 were determined. As a cellular model, LPS-stimulated MM6 cells that had been differentiated with calcitriol were used [20]. 6-Keto-$PGF_{1\alpha}$ was analyzed as COX-2-derived product after incubation of MM6 cells with 30 μM AA. As shown in Fig. 5A, BAs caused only marginal inhibition of COX-2 product synthesis in MM6 cells (IC_{50} >100 μM), whereas celecoxib (10 μM) or diclofenac (10 nM, used as positive controls) clearly suppressed the formation of 6-keto-$PGF_{1\alpha}$. To assess direct inhibition of COX-2 in cell-free assays, isolated human recombinant COX-2 was incubated with AA in the presence of GSH that leads to 12-HHT as major COX-2 product [27]. All BAs, suppressed COX-2 product synthesis Fig. 5B), although the potencies (IC_{50} > 100 μM) were modest as compared to celecoxib (IC_{50} approximately 10 μM, not shown). Note that also α-amyrin (100 μM) slightly affected COX-2 product synthesis (82.2 ± 1.6% of control). Hence, BAs may be regarded as poor inhibitors of COX-2. Attempts to precipitate COX-2 from lysates of MM6 cells (in analogy to the pull-down experiments for COX-1) failed and incubation of isolated COX-2 with BA- or KBA-seph bound only traces of COX-2 protein (data not shown).

3.5. *Docking of BAs into X-ray structures of COX enzymes*

Automated molecular docking of the BAs was performed to find potential binding modes for each BA within the active sites of the COX enzymes. As a test, we successfully re-docked the known co-crystallized inhibitors BFL (COX-1) and SC-558 (COX-2). Regarding COX-1, the RMSD for the inhibitor BFL was 0.34 ± 0.02 Å. The acquired binding mode was identical to the X-ray structure, yielding an average Chemscore of 37.3 ± 0.9. Docking of AKBA into the same docking box resulted in an average score of 14.5 ± 2.0. Aβ-BA achieved a Chemscore of 18.5 ± 2.0, KBA 10.9 ± 1.3 and β-BA 15.9 ± 1.1. All four BAs showed the same orientation in the binding pocket (Fig. 6A and B). For COX-2, the RMSD for the inhibitor SC-558 was 0.49 ± 0.04 Å. The acquired binding mode was identical to the X-ray structure and yielded a Chemscore of 30.5 ± 1.0. Docking of BAs into this docking box (not shown) resulted in comparably low docking scores. Thus, the average docking score of AKBA was 2.0 ± 5.0, Aβ-BA achieved a Chemscore of 6.6 ± 4.5, KBA 8.6 ± 1.9, and β-BA 10.7 ± 2.4.

4. Discussion

Initial attempts to elucidate the molecular mechanisms underlying the anti-inflammatory properties of extracts of *Boswellia* species identified 5-LO as relevant target. Thus, *Boswellia* species extracts as well as isolated BAs reduced the formation of LTs in various leukocytes [5,12,13,15] and inhibited 5-LO activity in cell-free leukocyte preparations [12,14,15] or isolated 5-LO enzyme *in vitro* [15]. Together with the fact that LTs play pivotal roles in inflammatory and allergic reactions, it was generally accepted that inhibition of LT formation is the molecular basis of the anti-inflammatory effects of BAs. Here, we show for the first time that BAs, in addition to 5-LO, also suppress the activity of COX enzymes by direct interactions.

Inhibition of COX-1 by BAs has long been excluded. Safayhi et al. showed that 11-keto-BAs (i.e. AKBA) inhibit 5-LO, whereas 11-methylene-BAs were hardly active [5,12,15]. In order to demonstrate a selectivity of BAs for 5-LO, the authors addressed the related enzymes p12-LO and COX-1, however, instead of AKBA, the 11-keto-free Aβ-BA was utilized for this purpose [5,17]. In fact, Aβ-BA is a poor inhibitor of COX-1 (ref. [5] and this study) and failed to inhibit p12-LO [6]. However, AKBA is a potent blocker of COX-1 in intact human platelets as well as of the isolated COX-1 enzyme. At least in intact platelets, the 11-keto moiety plays a critical role in the interference with COX-1 product synthesis, but also the 3-*O*-acetyl group seemingly is essential, since KBA suppressed COX-1 activity less efficient. Similar structure–activity

relationships are true for interference with 5-LO product formation [12]. Also for modulation of MAPK activation and Ca^{2+} mobilization in PMNL [40] and in monocytic cells [4], or for inhibition of the NFκB pathway [41], AKBA was the most potent BA analogue. Along these lines, we recently found that p12-LO is potently inhibited by AKBA, whereas the 11-methylene BAs rather stimulated this enzyme [6]. Interestingly, when the potencies of AKBA for inhibition of p12-LO (IC_{50} = 17 μM [6]), COX-1 (IC_{50} = 32 μM) and 5-LO (IC_{50} = 16–50 μM [2]) in cell-free assays are compared, there are actually only small differences apparent.

It should be noted that for inhibition of 5-LO activity quite inconsistent IC_{50} values between 1.5 up to 50 μM were reported [5,12,14,42]. These discrepancies seemingly depend on the different experimental settings (intact cells or cell-free assays, species, cell type, AA concentration, etc.) applied in the respective study. Also for COX-1, the efficacy of AKBA in intact cells (IC_{50} = 6–23 μM) is somewhat higher than in cell free assays (IC_{50} = 32 μM). It is possible that AKBA, due to its high hydrophobicity accumulates in cells or at least enriches in the subcellular locale(s) (membrane compartments) where activated COX-1 resides. Moreover, in intact platelets, the efficacy of AKBA depended on the stimulus (e.g. thrombin vs. A23187), and AKBA was most efficient to inhibit COX-1 when platelets were activated by A23187 (IC_{50} = 6 μM). It is unclear why BAs lacking the 11-keto moiety (β-BA and Aβ-BA) fail to potently block COX-1 activity under these conditions. Also for inhibition of 5-LO, AKBA was most potent in cells challenged by A23187, whereas β-BA and Aβ-BA were hardly efficient [5,42]. Possibly, the prominent increase in $[Ca^{2+}]_i$ evoked by A23187 or A23187-induced alterations of membrane characteristics may govern the cellular uptake and/or the interference of 11-keto-BAs with COX-1 (and 5-LO).

Besides the inhibition of product synthesis, a direct interference of BAs with COX-1 could be visualized by our protein pull-down approach using immobilized β-BA or KBA. Since the binding was reversed by AA or ibuprofen, we suggest that BAs bind to the active site of COX-1. In addition to COX-1, we previously showed that p12-LO is efficiently precipitated by KBA-Seph [6], but we were unable to demonstrate binding of 5-LO to immobilized KBA (KBA-Seph) [6]. Therefore, AKBA should not be regarded as a selective inhibitor of 5-LO, since it also almost equally well inhibits COX-1 and p12-LO, implying rather pleiotropic effects of BAs on enzymes within the AA cascade. Along these lines, COX-1/2 as well as 5- and 12-LO share the ability to bind and to oxygenate the same substrate (i.e. AA), and for 5-LO, AKBA was able to compete with AA for binding to the enzyme [16]. Similarly, inhibition of COX-1 by AKBA was reduced by increasing the amount of AA, and AA prevented binding of COX-1 to immobilized KBA. Regarding the chemical structure (Fig. 1), AKBA represents a lipophilic (fatty) acid that may fit into AA-binding sites of respective enzymes. Moreover, BAs were shown to inhibit the activity of various cytochrome P450 (CYP) enzymes (IC_{50} = 5–10 μM) [43] that can also metabolize AA, leading to epoxyeicosatrienoic acids [44].

Our docking results confirm the interaction of BAs with COX-1. All four BAs docked into the active site of COX-1, which might be achieved *via* van der Waals interactions complemented with hydrogen bonds formed by the hydroxyl or the acetyl-group with Y355 and R120. The carboxyl-group of the BAs is oriented towards the entry of the binding pocket, which might enable hydrophilic interactions with the solvent. The positive Chemscore values for the BAs indicate favorable binding to the active site of COX-1, even though they fail to explain the different IC_{50}-values measured. Noteworthy, the obtained Chemscore values are smaller than for the co-crystallized structures BFL (and SC-558). Relatively higher Chemscores were obtained for binding of the BAs to COX-1 over COX-2, correlating to the higher sensitivity of COX-1 towards BAs. Collectively, the docking study suggests favorable binding of BAs to COX-1, yielding similar binding modes as a well-recognized inhibitor. However, the latter might also arise from the fact that rigid inhibitor-bound protein conformations were employed for automated ligand docking. Fully flexible ligand–receptor docking allowing for induced-fit effects might come up with different results.

In view of the comparably low plasma concentrations of various BAs obtained after oral intake of a single dose of 1200–1600 mg of *Boswellia* species preparations (2–32 μM BAs [45]), one may speculate that the moderate COX-2 inhibition by BAs may be not of pharmacological relevance. It is well established that the inducible COX-2 has a superior role in inflammation over COX-1, visualized also by the high effectiveness of selective COX-2 inhibitors whereas COX-1 selective compounds are poorly anti-inflammatory [9]. However, the interference of AKBA with COX-1, probably in combination with inhibition of 5-LO, may contribute to the beneficial effects in arthritis disease models including Freund's adjuvant- or papaya latex-induced rat paw edema and inflammation, carrageenan- or dextran-induced edema in rats or mice, and osteoarthritis of dogs (reviewed in [1,2]). But still, interference of BAs with other known (NFκB route [3], MAPK pathway [4], p12-LO [6], HLE [7]) or other unknown targets may contribute in this regard.

In conclusion, despite the long appreciated opinion about AKBA and its selectivity for 5-LO, AKBA also interferes with COX-1, and as shown before, also with other AA-metabolizing enzymes (i.e. p12-LO [6] and CYPs [43]). Comparative studies with well-recognized COX-1 inhibitors revealed that AKBA is about equipotent to ibuprofen and aspirin in COX-1 inhibition. Possibly, the beneficial actions of AKBA-containing medication in the treatment of pain and inflammatory disorders, in particular arthritis, could also be related to the suppression of the biosynthesis of PGs, in addition to intervention with LTs.

Acknowledgements

The authors would like to acknowledge the financial support by Pharmasan GmbH (Freiburg, Germany) and by the Deutsche Forschungsgemeinschaft. We thank Bianca Jazzar for expert technical assistance. The Proteom Centrum Tübingen is supported by the Ministerium für Wissenschaft und Kunst, Landesregierung Baden-Württemberg.

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