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Arzanol, a prenylated heterodimeric phloroglucinyl pyrone, inhibits eicosanoid biosynthesis and exhibits anti-inflammatory efficacy *in vivo*

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ABSTRACT

Based on its capacity to inhibit *in vitro* HIV-1 replication in T cells and the release of pro-inflammatory cytokines in monocytes, the prenylated heterodimeric phloroglucinyl α-pyrone arzanol was identified as the major anti-inflammatory and anti-viral constituent from *Helichrysum italicum*. We have now investigated the activity of arzanol on the biosynthesis of pro-inflammatory eicosanoids, evaluating its anti-inflammatory efficacy *in vitro* and *in vivo*. Arzanol inhibited 5-lipoxygenase (EC 7.13.11.34) activity and related leukotriene formation in neutrophils, as well as the activity of cyclooxygenase (COX)-1 (EC 1.14.99.1) and the formation of COX-2-derived prostaglandin (PG)E_2 *in vitro* (IC_{50} = 2.3–9 μM). Detailed studies revealed that arzanol primarily inhibits microsomal PGE_2 synthase (mPGES)-1 (EC 5.3.99.3, IC_{50} = 0.4 μM) rather than COX-2. In fact, arzanol could block COX-2/mPGES-1-mediated PGE_2 biosynthesis in lipopolysaccharide-stimulated human monocytes and human whole blood, but not the concomitant COX-2-derived biosynthesis of thromboxane B_2 or of 6-keto $PGF_{1\alpha}$, and the expression of COX-2 or mPGES-1 protein was not affected. Arzanol potently suppressed the inflammatory response of the carrageenan-induced pleurisy in rats (3.6 mg/kg, i.p.), with significantly reduced levels of PGE_2 in the pleural exudates. Taken together, our data show that arzanol potently inhibits the biosynthesis of pro-inflammatory lipid mediators like PGE_2 *in vitro* and *in vivo*, providing a mechanistic rationale for the anti-inflammatory activity of *H. italicum*, and a rationale for further pre-clinical evaluation of this novel anti-inflammatory lead.

1. Introduction

Inflammation is a complex biological response to harmful stimuli required to remove an offensive agent and to initiate the healing process. However, chronic inflammation can lead to diseases (e.g., rheumatoid arthritis, atherosclerosis, and autoimmune disorders) that still represent unmet clinical needs. The prostanoids and leukotrienes (LTs) formed from arachidonic acid (AA) via the cyclooxygenase (COX)-1/2 (EC 1.14.99.1) and 5-lipoxygenase (5-LO) (EC 7.13.11.34) pathway, respectively, mediate inflammatory responses, chronic tissue remodelling, cancer, asthma, and autoimmune disorders, but also possess homeostatic functions in the gastrointestinal tract, uterus, brain, kidney, vasculature, and host defence [1]. In fact, the clinical use of non-steroidal anti-inflammatory drugs (NSAIDs), a class of drugs that block formation of all prostanoids, is hampered by severe side effects including gastrointestinal injury, renal irritations, and

Abbreviations: AA, arachidonic acid; COX, cyclooxygenase; cPL, cytosolic phospholipase; DMSO, dimethyl sulfoxide; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FCS, fetal calf serum; FLAP, 5-lipoxygenase-activating protein; 12-HHT, 12(*S*)-hydroxy-5-*cis*-8,10-*trans*-heptadecatrienoic acid; IL, interleukin; 5-LO, 5-lipoxygenase; LPS, lipopolysaccharide; LT, leukotriene; mPGES, microsomal prostaglandin E_2 synthase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NFκB, nuclear factor kappa beta; NSAIDs, non steroidal anti-inflammatory drugs; PBS, phosphate-buffered saline; PBMC, peripheral blood mononuclear cells; PG, prostaglandin; PGC buffer, PBS pH 7.4 containing 1 mg/ml glucose and 1 mM $CaCl_2$; TBH, *tert*-butyl hydroperoxide.

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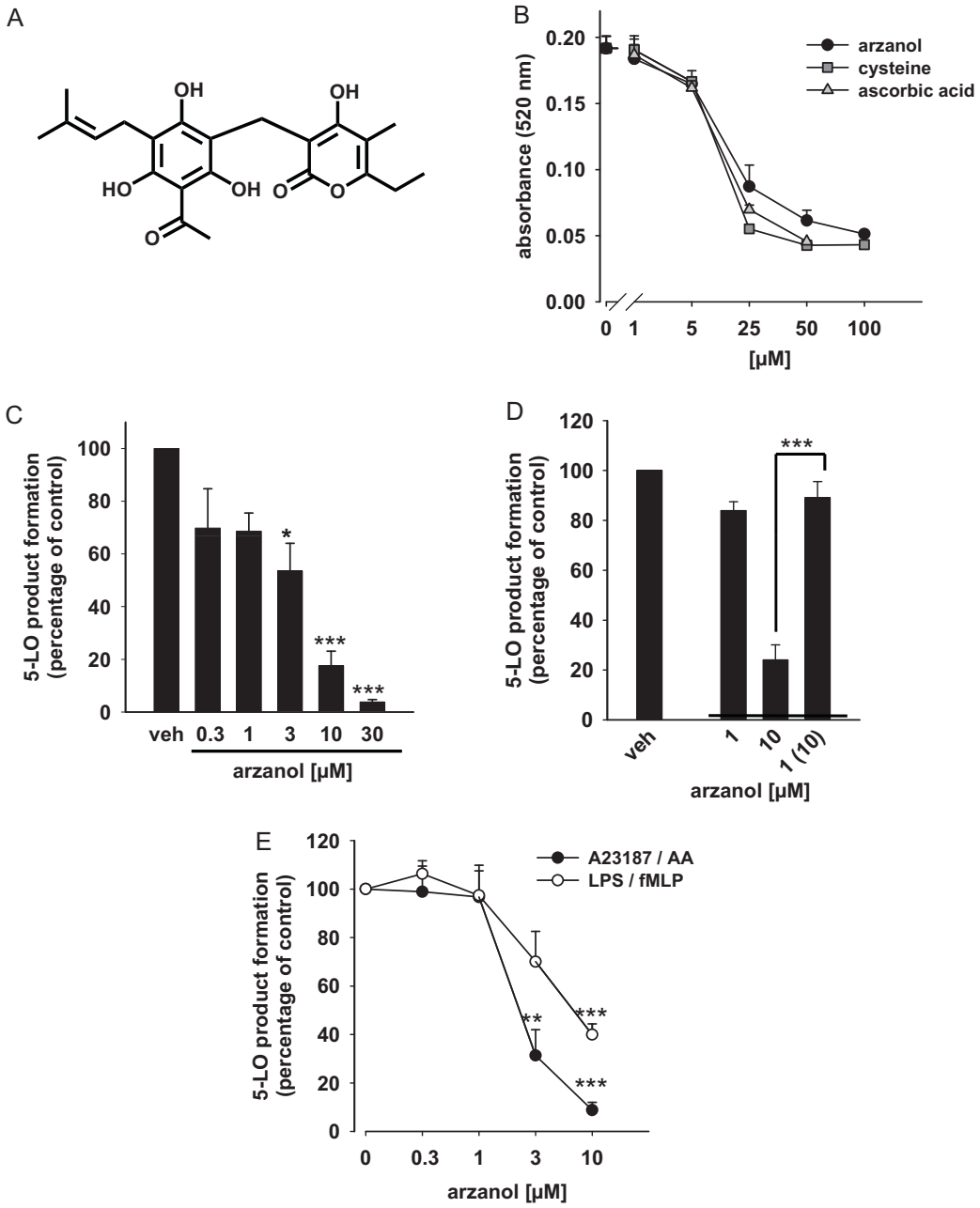

Fig. 1. Radical scavenging activity of arzanol and effects on 5-LO product formation. (A) Chemical structure of arzanol. (B) Radical scavenging activity of arzanol. The assay was performed as described in the method section. (C) Inhibition of 5-LO activity in a cell-free assay. Semi-purified human recombinant 5-LO (0.5 μg/ml) was pre-incubated with arzanol or vehicle (veh, DMSO) and 1 mM ATP for 10 min on ice, pre-warmed for 30 s at 37 °C, and 5-LO product formation was started by addition of 2 mM $CaCl_2$ and 20 μM AA. (D) Reversibility of 5-LO inhibition. Semi-purified human recombinant 5-LO (5 μg/ml) was pre-incubated with 10 μM arzanol or vehicle (veh, DMSO) for 10 min on ice. Then, an aliquot was diluted 10-fold with assay buffer, 1 mM ATP was added, pre-warmed for 30 s at 37 °C, and 5-LO product formation was started by addition of 2 mM $CaCl_2$ and 20 μM AA. As controls, 0.5 μg/ml 5-LO was pre-incubated with 1 or 10 μM arzanol (or vehicle, DMSO) and 5-LO product formation was analyzed as described above. (E) Inhibition of 5-LO activity in a cell-based assay. Neutrophils (5×10^6 cells/ml) were pre-incubated with the indicated concentrations of arzanol or vehicle (veh, DMSO) for 15 min. 5-LO product formation was initiated by addition of 2.5 μM A23187 plus 20 μM AA or by priming with LPS (1 μg/ml) and addition of 0.3 units Ada, and after 30 min 100 nM fMLP was added. The 100% values correspond to an average of 550 ng/ml 5-LO products for semi-purified enzyme, and 335 ng/ml (A23187/AA) or 250 pg/ml (LPS/fMLP) for intact cells, respectively. Data are given as mean + S.E., $n = 3–4$, $^{*}p < 0.05$, $^{**}p < 0.01$ or $^{***}p < 0.001$ vs. vehicle (0.1% DMSO) control, ANOVA + Tukey HSD *post hoc* tests.

cardiovascular risks [2]. Therefore, anti-inflammatory agents interfering with eicosanoid biosynthesis require a well-balanced pharmacological profile to minimize these on-target side effects [2]. Current anti-inflammatory research aims at identifying compounds that can suppress the massive formation of pro-inflammatory prostaglandin (PG)E_2 without affecting homeostatic PGE_2 and PGI_2 synthesis, and also inhibit LT formation [3,4]. While there is no shortage of anti-inflammatory natural products that suppress eicosanoid generation by inhibiting 5-LO and/or COX enzymes [5,6], the activity of most of them has only been evaluated in terms of expression and/or activity of COX enzymes and 5-LO *in vitro*, and high concentrations, unrealistic to translate in a clinical setting, are often required for activity [5]. Moreover, in many cases the anti-inflammatory activity was not evaluated *in vivo*, or, if so, it was not clearly related to an interference with the biosynthesis of eicosanoids.

Arzanol, a prenylated heterodimeric phloroglucinyl α-pyrone (Fig. 1A), was identified as the major anti-inflammatory constitu-

ent of *Helichrysum italicum* (*H. italicum*), a Mediterranean plant used in folk medicine to treat inflammatory diseases and infections [7,8]. In fact, extracts of *H. italicum* have been reported to exhibit anti-oxidant [7], anti-bacterial [9], anti-fungal [10] and anti-viral [11] effects. Extensive clinical research on the activity of extracts from *H. italicum* to manage inflammatory conditions was carried out by the Italian physician Leonardo Santini in the 40s and 50s. However, his findings were published in little known Journals, and, after his death, were largely overlooked [8]. Arzanol showed anti-oxidant activity in various systems of lipid peroxidation *in vitro*, and protected VERO cells against *tert*-butyl hydroperoxide (TBH)-induced oxidative stress [12]. Moreover, arzanol inhibited nuclear factor kappa beta (NFκB) activation and HIV-1 replication in T cells ($IC_{50} \sim 5$ μg/ml and 5 μM, respectively) as well as the release of pro-inflammatory mediators such as interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor (TNF)α, and PGE_2 in lipopolysaccharide (LPS)-stimulated monocytes [8]. Nevertheless, additional molecular target(s) of arzanol may exist and its anti-inflammatory efficacy *in vivo* remains to be investigated. To this aim, we have investigated the activity of arzanol on the biosynthesis of prostanoids and LTs, and we have evaluated its anti-inflammatory efficacy *in vitro* and *in vivo*. We show that arzanol potently inhibits the inducible microsomal PGE_2 synthase (mPGES)-1 (EC 5.3.99.3), COX-1, and 5-LO in various test systems with IC_{50} values between 0.4 and 9 μM, and significantly reduces the inflammatory response and the PGE_2 levels in the carrageenan-induced pleurisy in rats. These observations validate arzanol as a novel anti-inflammatory chemotype worth further development.

2. Materials and methods

2.1. Reagents

Arzanol was isolated from *H. italicum* as described [8], dissolved in dimethyl sulfoxide (DMSO) and kept in the dark at −20 °C, and freezing/thawing cycles were kept to a minimum. Arzanol was found to be stable in neutral medium in polar solvents. Thus, after 96 h at room temperature in DMSO, methanol or acetone, no degradation could be observed (NMR evidence). For animal studies, arzanol was dissolved in DMSO and diluted with saline achieving a final DMSO concentration of 4%. The thromboxane synthase inhibitor CV4151 was a generous gift by Dr. S. Laufer (University of Tuebingen, Germany). The mPGES-1 inhibitor 2-(2-chlorophenyl)-*1H*-phenanthro[9,10-d]-imidazole (MD52) was synthesized according to [13] and was a generous gift by Dr. M. Schubert-Zsilavecz (University Frankfurt, Germany). The antibody against human mPGES-1 was from Cayman Chemical (Ann Arbor, MI), the antibody against COX-2 was obtained from Enzo Life Sciences (Loerrach, Germany).

Materials used: DMEM/High Glucose (4.5 g/l) medium, penicillin, streptomycin, trypsin/EDTA solution, PAA Laboratories (Linz, Austria); λ-Carrageenan type IV isolated from *Gigartina aciculaire* and *Gigartina pistillata* and indomethacin were purchased from Sigma–Aldrich (Milan, Italy); PGH_2, Larodan (Malmö, Sweden); 11β-PGE_2, PGB_1, MK-886 (3-[1-(4-chlorobenzyl)-3-t-butyl-thio-5-isopropylindol-2-yl]-2,2-dimethylpropanoic acid), 6-keto $PGF_{1\alpha}$, Cayman Chemical (Ann Arbor, MI); Ultima Gold™ XR, Perkin Elmer (Boston, MA); [^{3}H]-PGE_2 was from PerkinElmer Life Sciences (Milan, Italy) and PGE_2 antibody from Sigma–Aldrich (Milan, Italy). All other chemicals were obtained from Sigma–Aldrich (Deisenhofen, Germany) unless stated otherwise.

2.2. Cells and cell viability assay

A549 cells were cultured in DMEM/High Glucose (4.5 g/l) medium supplemented with heat-inactivated fetal calf serum (FCS, 10%, v/v), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37 °C in a 5% CO_2 incubator. After 3 days, confluent cells were detached using 1× trypsin/EDTA solution and reseeded at 2×10^6 cells in 20 ml medium in 175 cm^2 flasks.

Blood cells were freshly isolated from leukocyte concentrates obtained at the Blood Center of the University Hospital Tuebingen (Germany) as described [14]. In brief, venous blood was taken from healthy adult donors that did not take any medication for at least 7 days, and leukocyte concentrates were prepared by centrifugation (4000 × *g*, 20 min, 20 °C). Cells were immediately isolated by dextran sedimentation and centrifugation on Nycoprep cushions (PAA Laboratories, Linz, Austria). Platelet-rich-plasma was obtained from the supernatants, mixed with phosphate-buffered saline (PBS) pH 5.9 (3:2, v/v), centrifuged (2100 × *g*, 15 min, room temperature), and the pelleted platelets were resuspended in PBS pH 5.9/0.9% NaCl (1:1, v/v). Washed platelets were finally resuspended in PBS pH 7.4 and 1 mM $CaCl_2$. Neutrophils were immediately isolated from the pellet after centrifugation on Nycoprep cushions, and hypotonic lysis of erythrocytes was performed as described [14]. Cells were finally resuspended in PBS pH 7.4 containing 1 mg/ml glucose and 1 mM $CaCl_2$ (PGC buffer) (purity > 96–97%). For isolation of human monocytes, peripheral blood mononuclear cells (PBMC) after centrifugation on Nycoprep cushions were washed three times with cold PBS. Then, monocytes were separated by adherence for 1 h at 37 °C to culture flasks (Greiner, Nuertingen, Germany; cell density was 2×10^7 cells/ml of RPMI 1640 medium containing 2 mM L-glutamine and 50 μg/ml penicillin/streptomycin), which finally gave a purity of >85%, defined by forward- and side-light scatter properties and detection of the CD14 surface molecule by flow cytometry (BD FACS Calibur, Rockville, MD). Monocytes were finally resuspended in ice-cold PBS plus 1 mg/ml glucose or in PGC buffer.

Cell viability was assessed by trypan blue staining and light microscopy as well as by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, A549 cells (4×10^5, plated into a 75 cm^3 cell culture flask and grown for 72 h) or human PBMC (10^6 cells/ml) were incubated at 37 °C and 5% CO_2. Then, arzanol was added and the incubation was continued for 24 h before cell viability was determined. Treatment with arzanol (10 or 30 μM) for 24 h did not significantly reduce cell viability in both cell cultures, excluding acute cytotoxic effects in the cellular assays used in this study. However, for A549 cells, incubation with 50 μM arzanol led to an increase in the ratio of dead cells to total number of cells (80% dead cells), whereas for the PBMC population no significant reduction of cell viability was evident at 50 μM arzanol (not shown).

2.3. Animals

Male adult Wistar Han rats (200–220 g, Harlan, Milan, Italy) were housed in a controlled environment and provided with standard rodent chow and water. Animal care complied with Italian regulations on protection of animals used for experimental and other scientific purpose (Ministerial Decree 116192) as well as with the European Economic Community regulations (Official Journal of E.C. L 358/1 12/18/1986).

2.4. DPPH assay

The radical scavenger capability was assessed by measuring the reduction of the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). Briefly, an ethanol solution of a sample at various concentrations (100 μl) was mixed with an acetate-buffered (pH 5–6.5) DPPH solution (final concentration 50 μM). After incubation for 30 min in the dark the absorbance of the mixture was measured at 520 nm.

2.5. Expression of human recombinant 5-LO, preparation of homogenates and 40 000 × g supernatants, and semi-purification of 5-LO

E. coli MV1190 was transformed with pT3-5-LO plasmid, and recombinant 5-LO protein was expressed at 27 °C as described [15]. Cells were lysed in 50 mM triethanolamine/HCl pH 8.0, 5 mM EDTA, soybean trypsin inhibitor (60 μg/ml), 1 mM phenylmethanesulfonyl fluoride, and lysozyme (500 μg/ml), homogenized by sonication (3 × 15 s), and centrifuged at 40,000 × *g* for 20 min at 4 °C. The 40,000 × *g* supernatant (S40) was applied to an ATP-agarose column to partially purify 5-LO as described previously [15]. S40 or semi-purified 5-LO were immediately used for 5-LO activity assays.

2.6. Determination of 5-LO product formation in cell-free systems

Aliquots of S40 or semi-purified 5-LO were diluted with ice-cold PBS containing 1 mM EDTA, and 1 mM ATP was added. Samples were pre-incubated with the test compounds as indicated. After 10 min at 4 °C, samples were pre-warmed for 30 s at 37 °C, and 2 mM $CaCl_2$ plus 20 μM AA was added to start 5-LO product formation. The reaction was stopped after 10 min at 37 °C by addition of 1 ml ice-cold methanol, and the formed metabolites were analyzed by RP-HPLC as described [16]. 5-LO products include the all-*trans* isomers of LTB_4 and 5(*S*)-hydro(pero)xy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid.

2.7. Determination of 5-LO product formation in intact cells

Freshly isolated neutrophils (1×10^7/ml PGC buffer) were pre-incubated with the test compounds for 15 min at 37 °C and 5-LO product formation was started by addition of 2.5 μM ionophore A23187 plus 20 μM AA. After 10 min at 37 °C, the reaction was stopped with 1 ml of methanol and 30 μl of 1 N HCl, and then, 200 ng PGB_1 and 500 μl PBS were added. Formed 5-LO metabolites were extracted and analyzed by HPLC as described [16]. 5-LO products include LTB_4 and its all-*trans* isomers, and 5(*S*)-hydro(pero)xy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid. Cysteinyl-LTs C_4, D_4, and E_4 were not detected, and oxidation products of LTB_4 were not determined.

For stimulation with LPS/fMLP, freshly isolated neutrophils (2×10^7/ml PGC buffer) were incubated with LPS (1 μg/ml). After 10 min, adenosine deaminase (Ada, 0.3 units/ml, in order to eliminate inhibitory extracellular adenosine) and the test compounds were added. After another 20 min, the reaction was initiated by 1 μM fMLP. After 5 min samples were placed on ice for 2 min, centrifuged (600 × *g*, 10 min), and LTB_4 was determined in the supernatant using a LTB_4 High Sensitivity EIA Kit (Assay Designs, Ann Arbor, MI) according to the manufacturer's protocol.

2.8. Determination of COX-1 activity in platelet homogenates and COX-2 activity in monocyte homogenates

Platelet homogenates (containing COX-1) were prepared by sonication (3 × 5 s at 4 °C) of freshly isolated platelets (10^8/ml PBS containing 1 mM EDTA). Monocytes were stimulated with LPS (10 μg/ml) for 20 h to induce COX-2 expression and cell homogenates were prepared by sonication (3 × 5 s at 4 °C; 4×10^6/ml PBS containing 1 mM EDTA). After addition of 5 mM glutathione and 5 μM haemoglobin, homogenates were pre-incubated with the test compounds for 4 min at room temperature and pre-warmed 1 min at 37 °C. COX reactions were started by addition of 5 μM AA, and after 5 min at 37 °C, 1 ml ice-cold methanol was added. The formed product 12(S)-hydroxy-5-*cis*-8,10-*trans*-heptadecatrienoic acid (12-HHT) was extracted and analyzed by HPLC as described [17].

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

Freshly isolated platelets (10^8/ml PBS containing 1 mM $CaCl_2$) were pre-incubated with the indicated agents for 10 min at room temperature and pre-warmed for 1 min at 37 °C. After addition of 5 μM AA and further incubation for 5 min at 37 °C, TXB_2 formed was quantified using a TXB_2 High Sensitivity EIA Kit (Assay Designs, Ann Arbor, MI), and 12-HHT was extracted and analyzed by HPLC as described [17].

2.10. Determination of 6-keto $PGF_{1\alpha}$ formation in intact A549 cells

The formation of 6-keto $PGF_{1\alpha}$ was determined in A549 cells according to [18]. A549 cells (2×10^6 cells) were incubated for 16 h at 37 °C and 5% CO_2, the medium was changed, and the cells were stimulated with IL-1β (1 ng/ml) for 48 h. After trypsination, cells were washed with PBS twice, and 2×10^6 cells were resuspended in 1 ml PBS containing $CaCl_2$ (1 mM), pre-incubated with the indicated compounds for 10 min at 37 °C, and 6-keto $PGF_{1\alpha}$ formation was initiated by addition of AA (1 μM). After 30 min at 37 °C, the reaction was stopped by cooling on ice. Cells were centrifuged (300 × *g*, 5 min, 4 °C), and the amount of released 6-keto $PGF_{1\alpha}$ was assessed by the High Sensitivity EIA Kit (Assay Designs, Ann Arbor, MI) according to the manufacturer's protocol.

2.11. Determination of PGE_2 formation in intact human monocytes

Formation of PGE_2 in intact cells was determined in monocytes according to Karlsson et al. [19]. In brief, freshly isolated monocytes were cultured in 12-well plates (density 10^6/ml per well) in RPMI medium containing 0.5% FCS and pre-treated with LPS (1 μg/ml) for 20 h. Cells were washed three times with PBS, and new medium was added for 30 min. After the medium was replaced again, cells were pre-incubated with the test compounds for 15 min. PGE_2 formation was initiated by addition of AA (1 μM). After 30 min, the amount of PGE_2 in the supernatant was measured using a PGE_2 High Sensitivity EIA Kit (Assay Designs, Ann Arbor, MI) according to the manufacturer's protocol.

2.12. SDS–PAGE and Western blot

Monocytes (5×10^6 cells) were resuspended in 50 μl PBS buffer pH 7.2, mixed with the same volume of 2× SDS/PAGE sample loading buffer (20 mM Tris–HCl, pH 8, 2 mM EDTA, 5% (m/v) SDS, and 10% (v/v) β-mercaptoethanol), 20 μl glycerol/0.1% bromophenol blue (1:1, v/v) and boiled for 5 min at 95 °C. Proteins were separated by SDS–PAGE. Correct loading of the gel and transfer of proteins to the nitrocellulose membrane was confirmed by Ponceau staining. After electroblotting to nitrocellulose membrane (GE Healthcare, Munich, Germany) and blocking with 5% BSA for 1 h at room temperature, membranes were washed and incubated with primary antibodies overnight at 4 °C. The membranes were washed and incubated with a 1:1000 dilution of alkaline phosphatase-conjugated immunoglobulin G for 1 h at room temperature. After washing, proteins were visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate.

2.13. Determination of PGE_2, 6-keto $PGF_{1\alpha}$, and TXB_2 formation in human whole blood

Peripheral blood from healthy adult volunteers, who had not received any medication for at least 2 weeks under informed consent, was obtained by venepuncture and collected in syringes

containing heparin (20 U/ml). Aliquots of whole blood (PGE_2: 0.8 ml, 6-keto $PGF_{1\alpha}$: 0.4 ml) were mixed with CV4151 (1 μM) and with aspirin (50 μM) for determination of PGE_2 and with CV4151 (1 μM) for analysis of 6-keto $PGF_{1\alpha}$, respectively. For determination of TXB_2, aliquots of whole blood (0.4 ml) were used without additives. A total volume of 1 ml or 0.5 ml was adjusted with sample buffer (10 mM potassium phosphate buffer pH 7.4, 3 mM KCl, 140 mM NaCl, and 6 mM D-glucose). After pre-incubation with the indicated compounds for 5 min at room temperature, the samples were stimulated with LPS (10 μg/ml) for 5 h at 37 °C. Prostanoid formation was stopped on ice, the samples were centrifuged (2300 × *g*, 10 min, 4 °C) and 6-keto $PGF_{1\alpha}$ and TXB_2 were quantified in the supernatant using High Sensitivity EIA Kits (Assay Designs, Ann Arbor, MI), respectively, according to the manufacturer's protocols. PGE_2 was determined as described [18]. In brief, the supernatant was acidified with citric acid (30 μl, 2 M), and after centrifugation (2300 × *g*, 10 min, 4 °C), solid phase extraction and separation by RP-HPLC was performed to isolate PGE_2. The PGE_2 peak (3 ml), identified by co-elution with the authentic standard, was collected, and acetonitrile was removed under a nitrogen stream. The pH was adjusted to 7.2 by addition of 10× PBS buffer pH 7.2 (230 μl) before PGE_2 contents were quantified using a PGE_2 High Sensitivity EIA Kit (Assay Designs, Ann Arbor, MI) according to the manufacturer's protocol.

2.14. Preparation of crude mPGES-1 in microsomes of A549 cells and determination of PGE_2 synthase activity

Preparation of A549 cells and determination of mPGES-1 activity was performed as described previously [18]. In brief, cells were treated with 1 ng/ml IL-1β for 48 h at 37 °C and 5% CO_2. After sonication, the homogenate was subjected to differential centrifugation at 10,000 × *g* for 10 min and 174,000 × *g* for 1 h at 4 °C. The pellet (microsomal fraction) was resuspended in 1 ml homogenization buffer (0.1 M potassium phosphate buffer pH 7.4, 1 mM phenylmethanesulfonyl fluoride, 60 μg/ml soybean trypsin inhibitor, 1 μg/ml leupeptin, 2.5 mM glutathione, and 250 mM sucrose), and the total protein concentration was determined. Microsomal membranes were diluted in potassium phosphate buffer (0.1 M, pH 7.4) containing 2.5 mM glutathione. Test compounds or vehicle were added, and after 15 min at 4 °C, the reaction (100 μl total volume) was initiated by addition of PGH_2 (20 μM, final concentration, unless stated otherwise). After 1 min at 4 °C, the reaction was terminated using stop solution (100 μl; 40 mM $FeCl_2$, 80 mM citric acid, and 10 μM of 11β-PGE_2 as internal standard). PGE_2 was separated by solid phase extraction and analyzed by RP-HPLC as described [18].

2.15. Carrageenan-induced pleurisy in rats

Arzanol (3.6 mg/kg) or indomethacin (5 mg/kg) were given i.p. 30 min before carrageenan. A group of male rats received the vehicle (DMSO, 4%, i.p.) 30 min before carrageenan. Rats were anaesthetized with enflurane 4% mixed with O_2, 0.5 l/min, N_2O 0.5 l/min and submitted to a skin incision at the level of the left sixth intercostal space. The underlying muscle was dissected, and saline (0.2 ml) or λ-carrageenan type IV 1% (w/v, 0.2 ml) was injected into the pleural cavity. The skin incision was closed with a suture, and the animals were allowed to recover. At 4 h after the injection of carrageenan, the animals were killed by inhalation of CO_2. The chest was carefully opened, and the pleural cavity was rinsed with 2 ml saline solution containing heparin (5 U/ml). The exudate and washing solution were removed by aspiration, and the total volume was measured. Any exudate that was contaminated with blood was discarded. The amount of exudates was calculated by subtracting the volume injected (2 ml) from the total volume recovered. Leukocytes in the exudates were resuspended in PBS and counted with an optical light microscope in a Burker's chamber after vital trypan blue staining.

The amounts of PGE_2, LTB_4 and 6-keto $PGF_{1\alpha}$ in the supernatant of centrifuged exudate (800 × *g* for 10 min) were assayed by radioimmunoassay (PGE_2) and enzyme immunoassay (LTB_4, 6-keto $PGF_{1\alpha}$), respectively (Cayman Chemical, Ann Arbor, MI) according to manufacturer's protocol. The results are expressed as ng per rat and represent the mean ± S.E. of 10 rats.

2.16. Statistics

Data are expressed as mean ± S.E. IC_{50} values are approximations determined by graphical analysis (linear interpolation between the points between 50% activity). The program Graphpad Instat (Graphpad Software Inc., San Diego, CA) was used for statistical comparisons. 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 (*) was considered significant.

3. Results

3.1. Effects of arzanol on 5-LO product formation

Many plant-derived 5-LO inhibitors are lipophilic reducing agents that act by uncoupling the redox cycle of the active-site iron of 5-LO or by radical scavenging activity [5]. In agreement with the result from previous studies [12], we could confirm the anti-oxidant properties of arzanol. Thus, arzanol caused a concentration-dependent reduction of DPPH with similar efficiency as well-recognized antioxidants such as L-cysteine or ascorbic acid (IC_{50} approx. 20 μM, Fig. 1B). Based on the anti-oxidant effect and the lipophilic structure, we hypothesized that arzanol could interfere with 5-LO, a redox-regulated dioxygenase. As shown in Fig. 1C, arzanol potently suppressed the activity of semi-purified 5-LO in a cell-free assay with an IC_{50} = 3.1 μM. Wash-out experiments demonstrated that this effect was reversible, as incubation of 5-LO with 10 μM arzanol and subsequent dilution to 1 μM (final concentration) abolished 5-LO inhibition (Fig. 1D).

Next, we evaluated the effects of arzanol on 5-LO activity in intact human neutrophils. A concentration-dependent inhibition of 5-LO product synthesis by arzanol was evident for cells stimulated with ionophore A23187 plus exogenous AA (20 μM) as well as for cells primed with LPS (1 μg/ml, 30 min) and then stimulated with 100 nM fMLP (IC_{50} = 2.9 and 8.1 μM, respectively, Fig. 1E). An interference with AA supply, e.g., by inhibition of $cPLA_2$ can be excluded, because for A23187-activated neutrophils exogenous AA was provided, and thus endogenous substrate supply was circumvented. Also, arzanol up to 10 μM failed to inhibit isolated recombinant human $cPLA_2$ whereas the pyrrolidine $cPLA_{2\alpha}$ inhibitor (5 μM) was active (not shown). The 5-LO inhibitor BWA4C (0.3 μM), used as reference compound, blocked 5-LO activity in all assays as expected (inhibition >90%, not shown). Together, arzanol is a direct inhibitor of 5-LO with efficiency also in intact cells.

3.2. Effects of arzanol on prostanoid formation

Prostanoids derived from the COX pathway are major inflammatory mediators formed by redox-sensitive dioxygenases, and thus we also evaluated the effects of arzanol on COX activity. As a robust test system for COX-1, we used human platelets pre-incubated with test compounds for 10 min and we measured the formation of 12-HHT and TXB_2 after addition of the COX substrate AA (5 μM). The formation of both 12-HHT and TXB_2 was

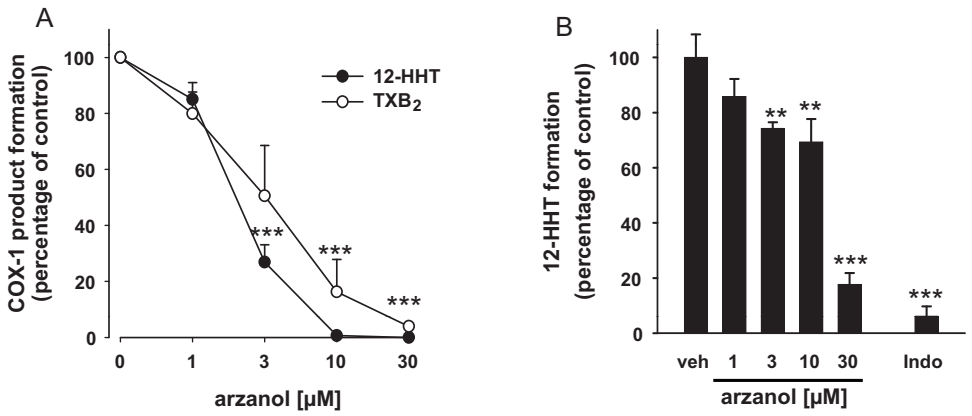

Fig. 2. Effects of arzanol on COX-1 activity. (A) COX-1 activity in human platelets. Platelets (10^8/ml PBS containing 1 mM $CaCl_2$) were pre-incubated with the indicated concentrations of arzanol or vehicle (veh, DMSO) for 5 min prior to stimulation with AA (5 μM). After another 5 min at 37 °C, the formation of 12-HHT and TXB_2 was assessed by HPLC and by ELISA, respectively. TXB_2 formed in the absence of test compounds averaged at 130 ng/ml (100%, control). The formation of 12-HHT was determined by RP-HPLC as described. 12-HHT formed in the absence of test compounds averaged at 56 ng/ml (100%, control). (B) COX-1 activity in platelet homogenates. Platelet homogenates were supplemented with 5 mM glutathione and 5 μM haemoglobin and pre-incubated with the test compounds or vehicle (veh, DMSO) for 4 min at RT and 1 min at 37 °C. Then, 5 μM AA was added, and after 5 min at 37 °C the amounts of 12-HHT formed were analyzed by HPLC. Data are given as mean + S.E., $n = 4$, $^{**}p < 0.01$ or $^{***}p < 0.001$ vs. vehicle (0.1% DMSO) control, ANOVA + Tukey HSD *post hoc* tests. Indo, indomethacin (10 μM).

concentration-dependently inhibited by arzanol with IC_{50} = 2.3 and 2.9 μM, respectively (Fig. 2A). Platelets also express the platelet-type 12-LO that converts AA to 12-H(P)ETE and we also observed a suppression of 12-HETE synthesis by arzanol at higher concentrations (IC_{50} approx. 20 μM, data not shown). Note that interference of arzanol with signalling pathways and release of AA as substrate can again be excluded since AA was supplied exogenously. In fact, arzanol inhibited COX-1 activity also in cell-free assays using lysates of human platelets as source of COX-1 enzyme (IC_{50} = 17.5 μM, Fig. 2B).

For analysis of modulation of COX-2 activity, human lung epithelial A549 cells were treated with IL-1β to induce COX-2 and to form the stable PGI_2 degradation product 6-keto $PGF_{1\alpha}$ (as biomarker for PGI_2 formation) from exogenous AA (1 μM) [18]. A contribution of COX-1 can be excluded in these cells because COX-1 is detectable neither at the protein nor at its corresponding mRNA level [12,20]. Arzanol moderately inhibited 6-keto $PGF_{1\alpha}$ formation, with an apparent $IC_{50} \geq 30$ μM (Fig. 3A). Higher concentrations of arzanol were not tested because at 50 μM, but not at 30 μM, arzanol significantly impaired cell viability (by 80% within 24 h, assessed by MTT assay, not shown). Along these lines, arzanol up to 30 μM failed to substantially inhibit COX-2 in a cell-free assay where human monocytes were used as enzyme source; indomethacin (10 μM) and the COX-2 selective celecoxib (5 μM) clearly blocked COX-2 activity (Fig. 3B).

In addition, we investigated the effects of arzanol on cellular COX-2-derived PGE_2 formation in intact human monocytes derived from peripheral blood that were stimulated with LPS for 24 h and subsequently PGE_2 was assessed by ELISA [19]. Monocyte-based PGE_2 analysis by ELISA is convenient and, in contrast to A549 cells, does not require preceding separation of prostanoids prior to ELISA or laborious pre-labelling with [^{3}H]AA [3]. Thus, IL-1β-treated A549 cells might produce substantial amounts of other prostanoids (e.g., 6-keto $PGF_{1\alpha}$) and related products that seemingly interfere with commercially available PGE_2 ELISA detection systems, see also [18]. The COX inhibitor indomethacin and the mPGES-1 inhibitor MD52 were used as controls. Arzanol significantly inhibited PGE_2 formation already at 3 μM and the IC_{50} was determined at 9 μM (Fig. 3C). This inhibitory effect was not related to an accompanied interference with the expression of COX-2 or mPGES-1 by arzanol (Fig. 3D). Along these lines, arzanol inhibited PGE_2 formation in human whole blood stimulated with LPS for 5 h, starting at 3 μM, and at 30 μM arzanol PGE_2 synthesis was repressed by about 50% (Fig. 4A). In parallel, the accompanied COX-2-mediated biosynthesis of TXB_2 (Fig. 4B) and the COX-2-derived 6-keto $PGF_{1\alpha}$ formation (Fig. 4C) were not significantly inhibited by arzanol. It is worth notice that the selective mPGES-1 inhibitor MD52 showed a comparable inhibitory profile, clearly reducing PGE_2 synthesis in LPS-treated monocytes and whole blood, but hardly affecting 6-keto $PGF_{1\alpha}$ in the respective assays. In contrast, indomethacin blocked the formation of the analyzed prostanoids in all assays, as expected. Taken together, arzanol obviously inhibits COX-1 activity but is a poor inhibitor of COX-2 derived prostanoids, except for PGE_2 which is clearly suppressed.

3.3. Effects of arzanol on mPGES-1

The suppression of PGE_2 formation by arzanol without concomitant inhibition of other COX-2-derived prostanoids led us to hypothesize that arzanol could interfere with mPGES-1. Microsomal preparations of IL-1β-stimulated A549 cells, used as source of mPGES-1 [21], were pre-incubated with test compounds for 15 min, and then, PGE_2 formation was initiated by addition of 20 μM PGH_2. The mPGES-1 inhibitor MK-886 (used as control) blocked PGE_2 formation with an IC_{50} of 2.1 μM (data not shown). Arzanol concentration-dependently inhibited PGE_2 formation with an IC_{50} = 0.4 μM (Fig. 5A) being superior over MK-886. Alteration of PGH_2 to lower (1 μM) or higher (50 μM) concentration caused only slight changes in the potency of arzanol (Fig. 5B), suggesting that mPGES-1 inhibition is largely independent of the substrate concentration.

To investigate whether arzanol inhibits PGE_2 synthesis in a reversible manner, wash-out experiments were carried out. Arzanol failed to efficiently block PGE_2 synthesis at 0.1 μM, whereas PGE_2 formation was efficiently inhibited at 1 μM (Fig. 5C). However, 10-fold dilution of the samples containing 1 μM arzanol restored mPGES-1 activity implying a reversible mode of inhibition. It appeared possible that arzanol could directly act on PGH_2 and thus inhibit non-enzymatic PGE_2 formation independent of mPGES-1. However, when the microsomal preparations of A549 cells were heat-inactivated (15 min/65 °C) the non-enzymatic PGE_2 formation was not affected by arzanol up to 10 μM (not shown).

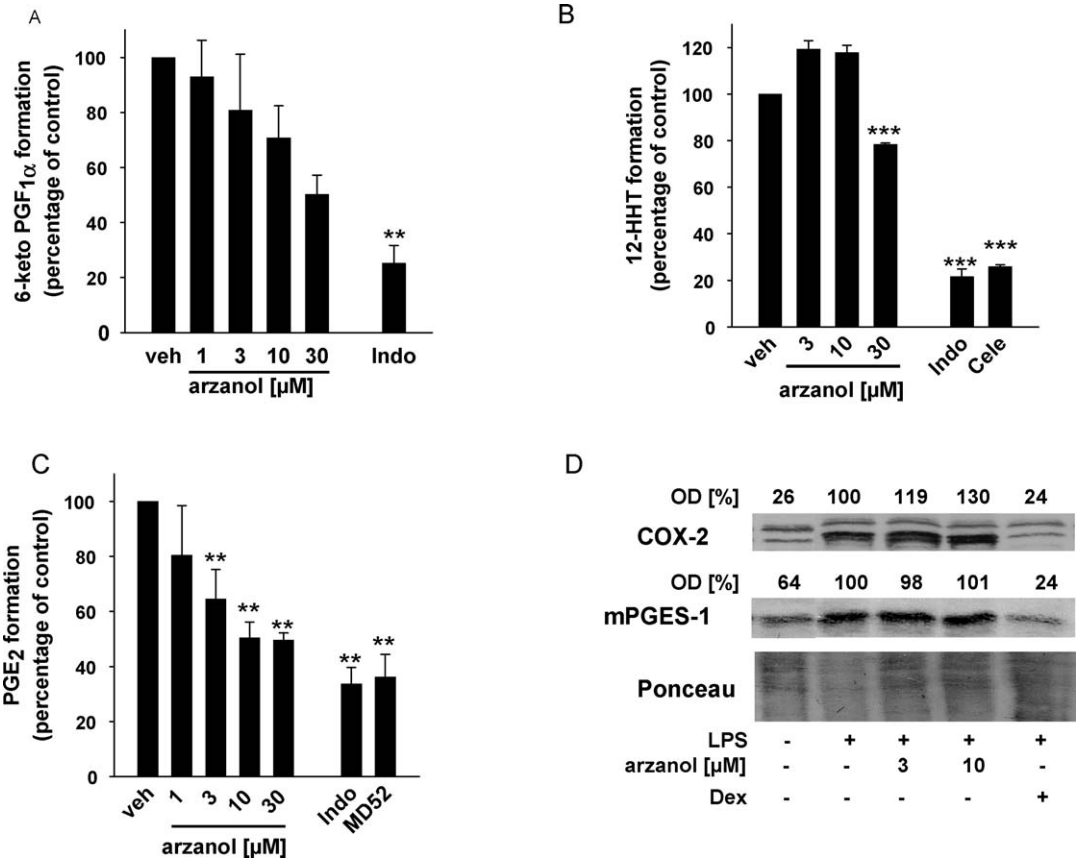

Fig. 3. Effects of arzanol on COX-2 activity. (A) 6-Keto $PGF_{1\alpha}$ formation as parameter of COX-2 activity. IL-1β-stimulated A549 cells (1×10^6/ml) were pre-incubated with arzanol, indomethacin (Indo, 10 μM) or vehicle (veh, DMSO) as indicated for 15 min at RT prior to addition of 1 μM AA. After 30 min at 37 °C the amount of 6-keto $PGF_{1\alpha}$ was assessed by ELISA as described. The 100% value corresponds to an average of 1.2 ng/ml 6-keto $PGF_{1\alpha}$. (B) COX-2 activity in monocyte homogenates. Monocytes were stimulated with LPS (10 μg/ml) for 20 h, cell homogenates were prepared, 5 mM glutathione and 5 μM haemoglobin were added, and pre-incubated with arzanol, indomethacin (Indo, 10 μM), celecoxib (Cele, 5 μM) or vehicle (veh, DMSO) for 4 min at RT and 1 min at 37 °C. Then, 5 μM AA was added and 12-HHT was analyzed by HPLC. (C) PGE_2 formation as parameter of COX-2 activity. Monocytes were pre-treated with LPS (1 μg/ml) for 20 h, cells were washed, and pre-incubated with arzanol, indomethacin (Indo, 10 μM), MD52 (2 μM) or vehicle (veh, DMSO) for 15 min. PGE_2 formation was initiated by addition of AA (1 μM), and after 30 min, PGE_2 in the supernatant was analyzed by ELISA. (D) Expression of COX-2 and mPGES-1. Monocytes (2×10^6 cells/ml RPMI plus 0.5% FCS) were incubated in the presence or absence of 10 μg/ml LPS together with arzanol, dexamethasone (1 μM) or vehicle (DMSO) for 20 h at 37 °C. Cells were harvested, lysed by addition of SDS/PAGE sample loading buffer and proteins were separated by SDS–PAGE and analyzed for COX-2 (upper panel) and mPGES-1 (lower panel) by WB. Ponceau S staining confirms equal loading of protein and transfer of proteins to the membrane. Data are given as mean + S.E., $n = 3$–4, $^{**}p < 0.01$ or $^{***}p < 0.001$ vs. vehicle (0.1% DMSO) control, ANOVA + Tukey HSD *post hoc* tests.

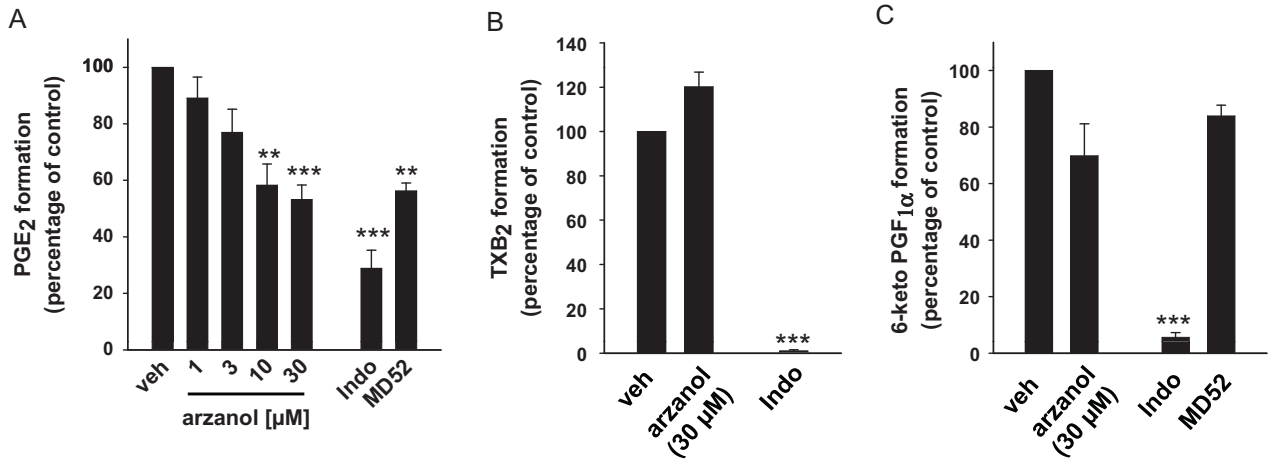

Fig. 4. Effects of arzanol on prostanoid formation in human whole blood. (A) PGE_2 formation. Heparinized human blood, treated with 1 μM thromboxane synthase inhibitor and 50 μM aspirin, was pre-incubated with arzanol, indomethacin (Indo, 50 μM), MD52 (6 μM) or vehicle (veh, DMSO) for 5 min at RT, and then, PGE_2 formation was induced by addition of 10 μg/ml LPS. After 5 h at 37 °C, PGE_2 was extracted from plasma and analyzed as described. The 100% value corresponds to an average of 1.9 ng/ml PGE_2, and 0.42 ng/ml PGE_2 were detected in unstimulated blood. (B) TXB_2 formation. Heparinized blood was pre-incubated with arzanol, indomethacin (Indo, 50 μM) or vehicle (veh, DMSO, w/o) for 5 min, then 10 μg/ml LPS were added and TXB_2 was analyzed after 5 h at 37 °C in the plasma by ELISA. The 100% value corresponds to an average of 58 ng/ml TXB_2. (C) 6-Keto $PGF_{1\alpha}$ formation. Heparinized human blood, treated with 1 μM thromboxane synthase inhibitor, was pre-incubated with arzanol, indomethacin (Indo, 50 μM), MD52 (6 μM) or vehicle (veh, DMSO) for 5 min at RT, prior to addition of 10 μg/ml LPS. After 5 min at 37 °C, 6-keto $PGF_{1\alpha}$ was directly analyzed in plasma by ELISA. The 100% value corresponds to an average of 14.5 ng/ml 6-keto $PGF_{1\alpha}$, and 2.3 ng/ml 6-keto $PGF_{1\alpha}$ were detected in unstimulated blood. Data are given as mean ± S.E., $n = 3$, $^{**}p < 0.01$ or $^{***}p < 0.001$ vs. vehicle (0.1% DMSO) control, ANOVA + Tukey HSD *post hoc* tests.

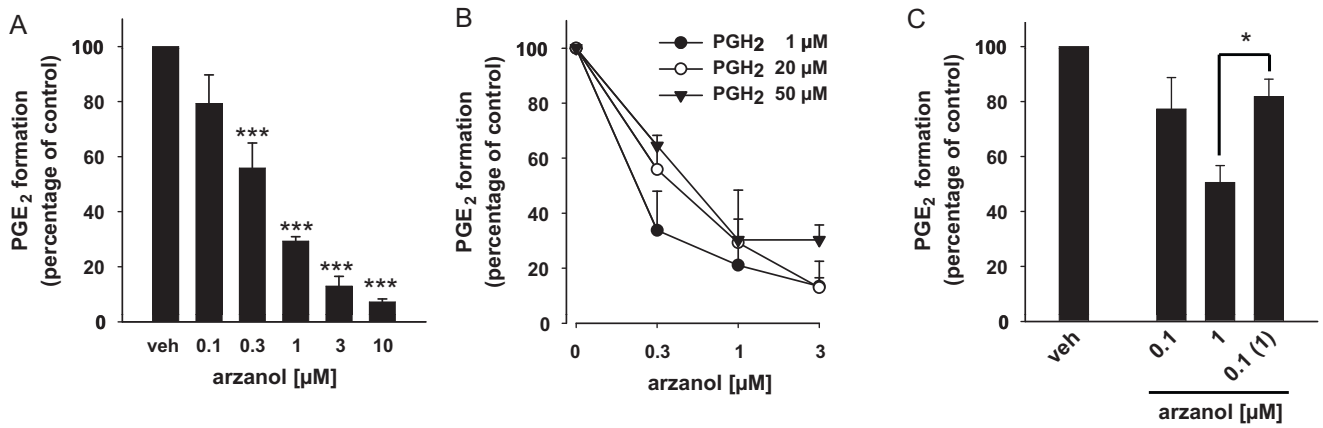

Fig. 5. Effects of arzanol on the activity of mPGES-1. (A) Concentration–response curve. Microsomal preparations of IL-1β-stimulated A549 cells were pre-incubated with arzanol or vehicle (veh, DMSO) for 15 min at 4 °C, and the reaction was started with 20 μM PGH_2. After 1 min at 4 °C, the reaction was terminated using a stop solution containing $FeCl_2$ and 11β-PGE_2 (1 nmol) as internal standard. The 100% value corresponds to an average of 0.8 nmol PGE_2. (B) The potency of arzanol for mPGES-1 inhibition was compared at 1, 20, and 50 μM PGH_2 as substrate. The amount of PGE_2 was quantified for 1 μM PGH_2 by use of a PGE_2 High Sensitivity EIA Kit according to the manufacturer's protocol; for 20 and 50 μM PGH_2, the PGE_2 was quantified by HPLC. The 100% values correspond to an average of 70 ng/ml (ELISA), or 1 and 5 nmol (HPLC) PGE_2 for 1, 20, and 50 μM PGH_2, respectively. (C) Reversibility of mPGES-1 inhibition by arzanol. Microsomal preparations of IL-1β-stimulated A549 cells were pre-incubated with 1 μM inhibitor for 15 min at 4 °C. An aliquot was diluted 10-fold to obtain an inhibitor concentration of 0.1 μM. For comparison, microsomal preparations were pre-incubated for 15 min with vehicle (veh, DMSO, w/o) or 0.1 μM arzanol, and then, 20 μM PGH_2 was added (no dilution). All samples were incubated for 1 min on ice, and PGE_2 formation was analyzed as described by HPLC. Data are given as mean + S.E., n = 3–4, $^{*}p < 0.05$ or $^{***}p < 0.001$ vs. vehicle (0.1% DMSO) control, ANOVA + Tukey HSD *post hoc* tests.

Table 1
Effect of arzanol on carrageenan-induced pleurisy in rats.

Treatment	Exudate volume (ml)	Inflammatory cells $\times 10^6$	PGE_2 (ng/rat)	LTB_4 (ng/rat)	6-Keto $PGF_{1\alpha}$ (ng/rat)
Vehicle	0.41 ± 0.019	46.5 ± 6.0	4.26 ± 0.67	1.22 ± 0.26	10.72 ± 1.43
Arzanol (3.6 mg/kg)	0.17 ± 0.05*** (59%)	24.0 ± 5.9* (48%)	2.27 ± 0.35* (47%)	0.84 ± 0.21 (31%)	7.79 ± 0.72 (27%)
Indomethacin (5 mg/kg)	0.10 ± 0.03*** (76%)	16.28 ± 2.30*** (65%)	0.21 ± 0.024*** (95%)	1.06 ± 0.20 (13%)	0.64 ± 0.09*** (94%)

Thirty minutes before intrapleural injection of carrageenan, rats (n = 10 per group) were treated i.p. with 3.6 mg/kg arzanol, indomethacin (5 mg/kg) or vehicle (DMSO 4%). Exudate volume, eicosanoids, and inflammatory cell accumulation in the pleural cavity were assessed 4 h after carrageenan injection. Data are expressed as mean ± SEM.
* $p < 0.05$ vs. vehicle.
*** $p < 0.001$ vs. vehicle.

3.4. Arzanol suppresses carrageenan-induced pleurisy in rats and inhibits eicosanoid bisosynthesis in vivo

The anti-inflammatory effectiveness of arzanol was assessed *in vivo* using carrageenan-induced pleurisy in rats. Injection of carrageenan into the pleural cavity of rats (DMSO 4% group) elicited an inflammatory response within 4 h, characterized by the accumulation of fluid that contained large numbers of inflammatory cells (Table 1). The dose of arzanol was chosen at 3.6 mg/kg, i.p., 30 min prior to carrageenan based on the experiences from previous animal studies using myrtucommulone or hyperforin with comparable efficiency to arzanol *in vitro* [22,23]. Arzanol inhibited the inflammatory response as demonstrated by the significant attenuation of exudate formation (59%) and cell infiltration (48%). Indomethacin (5 mg/kg) reduced exudate formation and cell infiltration by 75% and 65%, respectively (Table 1). In comparison with the corresponding exudates from DMSO-treated rats, exudates of arzanol-treated animals exhibited decreased PGE_2 levels (47% inhibition), whereas indomethacin almost completely suppressed PGE_2 (95%) as well as 6-keto $PGF_{1\alpha}$ (94%) levels as expected. In agreement with the moderate inhibition of COX-2-derived 6-keto $PGF_{1\alpha}$ formation in A549 cells or human whole blood (see above), arzanol also slightly reduced the levels of 6-keto $PGF_{1\alpha}$ (27% inhibition). On the other hand, indomethacin failed to significantly reduce LTB_4 levels which were lowered by arzanol (31% inhibition, seemingly due to direct inhibition of 5-LO).

4. Discussion

Arzanol was recently characterized as the major bioactive constituent of *H. italicum* with excellent anti-oxidant properties that inhibited the HIV-1 replication in T cells and the release of pro-inflammatory cytokines from stimulated monocytes, which was attributed to interference with the NFκB pathway [8,12]. We now report that arzanol potently inhibits the biosynthesis of PGE_2, TXB_2, and LTs which are pivotal mediators of inflammatory reactions, and we identified 5-LO, COX-1, and mPGES-1 as respective molecular targets of arzanol. Importantly, suppression of eicosanoid formation was evident also *in vivo* in the rat pleurisy model, and this was accompanied by significant anti-inflammatory efficacy. In the context of further preclinical studies, the marked inhibitory activity on LTs, COX-1-derived TXA_2 and COX-2-derived PGE_2, and the minor suppression of beneficial prostanoids (e.g., PGI_2, the instable precursor of 6-keto $PGF_{1\alpha}$) qualify arzanol a valuable anti-inflammatory candidate.

The role of eicosanoids in inflammation and allergy is well established, and drugs that interfere with either the biosynthesis (NSAIDs) or the action (cysteinyl LT receptor antagonists) of these lipid mediators are commonly used to treat inflammatory or allergic diseases [1,24]. Arzanol inhibits eicosanoid biosynthesis by interfering with 5-LO, mPGES-1 and COX-1, thus supporting its anti-inflammatory potential. In fact, one of the most advanced anti-inflammatory drug candidates, licofelone (in phase III clinical trials) [25] also essentially acts by blocking COX-1 and mPGES-1

and by inhibition of the 5-LO pathway [18,26]. Other dual inhibitors of mPGES-1 and 5-LO like the pirinixic acid derivative YS121 [27] or myrtucommulone [28] showed comparable effects on cellular eicosanoid biosynthesis as arzanol and both compounds also exhibited potent anti-inflammatory activities *in vivo* [27,23]. Moreover, arzanol blocks NFκB activation and release of pro-inflammatory cytokines (i.e., IL-1β, IL-6, IL-8 and TNFα) [8] that may potentiate its anti-inflammatory activity. Such multi-target properties may confer arzanol a favourable pharmacodynamic profile with possible advantages over compounds that inhibit only mPGES-1.

COX-1/2 and 5-LO are dioxygenases with iron in the AA-binding active site cycling between reduced and oxidized states during catalysis [29,30]. Anti-oxidants and/or radical scavengers with sufficient lipophilicity (e.g., polyphenols) are well-recognized inhibitors of 5-LO by keeping the non-heme iron in the inactive, reduced state or by interrupting the redox cycle [5]. Arzanol contains a phloroglucinol core with antioxidant features and showed antioxidant activity, protected linoleic acid against free radical attack in assays of autoxidation and EDTA-mediated oxidation, and inhibited TBH-induced oxidative stress in VERO cells [12]. Also in our DPPH assay, arzanol clearly acted as radical scavenger, and interference with the enzymatic activity of 5-LO is therefore reasonable for arzanol, as revealed by inhibition of semipurified 5-LO in a cell-free assay. In comparison to other well-recognized naturally occurring polyphenolic 5-LO inhibitors, arzanol (IC_{50} = 3.1 μM) is equipotent to caffeic acid (IC_{50} = 3.7 μM [31]) and even more potent than nordihydroguaiaretic acid (NDGA, IC_{50} = 28 μM [32]), magnolol (IC_{50} = 15–25 μM) [33] or the structurally related myrtucommulone (IC_{50} = 5 μM [34]). Importantly, arzanol inhibited 5-LO product formation also in intact neutrophils at similar concentrations as in the cell-free assay, implying that the reduced synthesis of 5-LO products in the cell might be related to direct interference of arzanol with the 5-LO enzyme. This however, supposes essentially unimpeded distribution of arzanol to the intracellular space but also multiple mechanisms in the cell-based system may contribute to suppression of 5-LO product synthesis. Since arzanol failed to inhibit $cPLA_2$ activity which is thought to be the major enzyme in neutrophils providing AA as substrate for 5-LO [35], suppression of 5-LO product synthesis at this level can be excluded. Furthermore, the efficiency of arzanol was comparable under conditions where substrate was provided from endogenous sources (stimulation with A23187 or LPS/fMLP) or, alternatively, supplemented exogenously (i.e., 20 μM AA).

NSAIDs essentially act by inhibiting COX-1 and COX-2 with different preferences for each individual drug [36,37]. Arzanol inhibited COX-1-mediated 12-HHT and TXB_2 synthesis in platelets treated with AA equally well whereas 12-LO product synthesis was less affected. Again, interference of arzanol with substrate supply can be excluded under these conditions and a direct effect of arzanol on COX-1 is assumed, supported by the inhibition of COX-1 activity in the cell-free assay, where other cellular parameters are not operative. Remarkably, arzanol (IC_{50} = 2.3–2.9 μM) is equipotent or even more potent than ibuprofen (IC_{50} = 3–70 μM [38,39]) or aspirin (IC_{50} = 3.2 μM [38]) in platelets, which are frequently used as COX inhibitors in the clinics [40].

In contrast to the consistent inhibition of COX-1 product formation, the suppression of COX-2-derived prostanoid generation by arzanol was heterogeneous. Arzanol blocked cellular PGE_2 synthesis and our data suggest that the selective suppression of PGE_2 is primarily due to interference with mPGES-1 but not with COX-2. Thus, arzanol hardly inhibited COX-2 activity in a cell-free assay ($IC_{50} \geq 30$ μM) under conditions where COX-1 was potently blocked. In IL-1β-treated A549 cells expressing solely COX-2, arzanol (30 μM) caused only modest inhibition of 6-keto $PGF_{1\alpha}$ formation. However, arzanol (3 μM) efficiently inhibited PGE_2 synthesis in LPS-stimulated monocytes, where also COX-2 is the predominant isoform under the respective experimental conditions [41]. Analysis of COX-2 and mPGES-1 protein in monocytes ruled out any genomic effects of arzanol on the expression level of these inflammatory end-points.

Divergent effects of arzanol on COX-2-derived prostanoid formation were also apparent when the compound was analyzed in the human whole blood assay. Neither TXB_2 synthesis nor formation of 6-keto $PGF_{1\alpha}$ were significantly reduced up to 30 μM arzanol in this assay, whereas under the same conditions arzanol suppressed PGE_2 synthesis at relatively low concentrations (3 μM) but reached a plateau at higher concentrations (30 μM) where about 50% PGE_2 still remained. Also the potent mPGES-1 inhibitor MD52 suppressed PGE_2 formation only by 50%, and failed to block TXB_2 and 6-keto-$PGF_{1\alpha}$, supporting mPGES-1 as the respective target of arzanol. Presumably, other PGES isoforms unaffected by MD52 or arzanol contribute to the basal PGE_2 generation in whole blood. On the other hand, the PGE_2 ELISA detection systems may recognize related (non-prostanoid) cross-reactive substances, supported by the incomplete suppression of PGE_2 formation by indomethacin in whole blood while TXB_2 and 6-keto-$PGF_{1\alpha}$ synthesis is effectively inhibited.

Arzanol reduced the inflammatory reaction in an *in vivo* model of acute inflammation, the carrageenan-induced rat pleurisy, with activity almost comparable to that of indomethacin. PGE_2 plays a central role in the early phase of carrageenan-induced pleurisy [42], and COX-inhibitors prevent the inflammatory response. Accordingly, lowering PGE_2 by inhibition of mPGES-1 may contribute to the anti-inflammatory properties of arzanol. As observed in the whole blood assay, arzanol was less potent than indomethacin in the reduction of PGE_2 levels, but still efficiently suppressed exudate formation and infiltration of inflammatory cells. In contrast to indomethacin, arzanol also reduced the levels of LTB_4 in the pleural exudates. Since 5-LO-derived metabolites cooperate with PGE_2 in the induction of pleurisy [43], inhibition of 5-LO may contribute to the anti-inflammatory effect of arzanol. Indeed, synergistic anti-inflammatory effects of so-called dual 5-LO/COX have been observed [25,44]. The concomitant blockade of LTB_4 formation is seemingly also responsible for the lack of gastric toxicity (the most troublesome side effect of NSAIDs) observed with 5-LO/COX dual inhibitors [44]. Future studies using animal models of gastric toxicity will reveal whether arzanol might exert its anti-inflammatory activity without gastric side effects. Taken together, our data show that arzanol potently inhibits the biosynthesis of pro-inflammatory lipid mediators like PGE_2 *in vitro* and *in vivo*, providing a mechanistic rationale for the anti-inflammatory activity of *H. italicum*, and a rationale for further pre-clinical evaluation of this novel anti-inflammatory lead.

Conflict of interest statement

None declared.

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