Arzanol; a prenylated heterodimeric phloroglucinyll pyrone; inhibits eicosanoid biosynthesis and exhibits anti-inflammatory efficacy

Julia Bauer, Andreas Koeberle, Friederike Dehm, Federica Pollastro, Giovanni Appendino, Hinnak Northoff, Antonietta Rossi, Lidia Sautebin, Oliver Werz

► To cite this version:

Julia Bauer, Andreas Koeberle, Friederike Dehm, Federica Pollastro, Giovanni Appendino, et al.. Arzanol; a prenylated heterodimeric phloroglucinyll pyrone; inhibits eicosanoid biosynthesis and exhibits anti-inflammatory efficacy. *Biochemical Pharmacology*, 2010, 81 (2), pp.259. 10.1016/j.bcp.2010.09.025 . hal-00649889

HAL Id: hal-00649889

<https://hal.science/hal-00649889>

Submitted on 9 Dec 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

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

Authors: Julia Bauer, Andreas Koeberle, Friederike Dehm, Federica Pollastro, Giovanni Appendino, Hinnak Northoff, Antonietta Rossi, Lidia Sautebin, Oliver Werz

PII: S0006-2952(10)00754-9
DOI: doi:10.1016/j.bcp.2010.09.025
Reference: BCP 10730

To appear in: *BCP*

Received date: 3-8-2010
Revised date: 29-9-2010
Accepted date: 29-9-2010

Please cite this article as: Bauer J, Koeberle A, Dehm F, Pollastro F, Appendino G, Northoff H, Rossi A, Sautebin L, Werz O, Arzanol; a prenylated heterodimeric phloroglucinyl pyrone; inhibits eicosanoid biosynthesis and exhibits anti-inflammatory efficacy *in vivo*, *Biochemical Pharmacology* (2010), doi:10.1016/j.bcp.2010.09.025

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

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

Running title: Arzanol inhibits eicosanoid biosynthesis

Julia Bauer^a, Andreas Koeberle^a, Friederike Dehm^{a/b}, Federica Pollastro^c, Giovanni Appendino^c, Hinnak Northoff^d, Antonietta Rossi^b, Lidia Sautebin^b, and Oliver Werz^{c,e*}

^a Department for Pharmaceutical Analytics, Pharmaceutical Institute, University of Tuebingen, Auf der Morgenstelle 8, D-72076 Tuebingen, Germany

E-mail addresses: julia.bauer@uni-tuebingen.de, andreas.koeberle@uni-tuebingen.de, friederike.dehm@uni-tuebingen.de, oliver.werz@uni-tuebingen.de

^b Department of Experimental Pharmacology, University of Naples "Federico II", Italy

E-mail addresses: antrossi@unina.it, sautebin@unina.it

^c Dipartimento di Scienze Chimiche, Alimentari, Farmaceutiche e Farmacologiche, Università del Piemonte Orientale, Italy

E-mail addresses: giovanni.appendino@pharm.unipmn.it,

federica.pollastro@pharm.unipmn.it

^d Institute for Clinical and Experimental Transfusion Medicine, University Medical Center Tuebingen, Hoppe-Seyler-Straße 3, 72076 Tuebingen, Germany

E-mail address: hinnak.northoff@med.uni-tuebingen.de

^e Chair of Pharmaceutical/Medicinal Chemistry, Institute of Pharmacy, University of Jena, Philosophenweg 14, D-07743 Jena, Germany. E-mail address: oliver.werz@uni-jena.de

*** Corresponding author:** Dr. Oliver Werz, Chair of Pharmaceutical/Medicinal Chemistry, Institute of Pharmacy, University of Jena, Philosophenweg 14, D-07743 Jena, Germany. Phone: +49-3641-949801; Fax: +49-3641-949802; e-mail: oliver.werz@uni-jena.de

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 antiviral 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)E₂ *in vitro* (IC₅₀ = 2.3 - 9 μ M). Detailed studies revealed that arzanol primarily inhibits microsomal PGE₂ synthase (mPGES)-1 (EC 5.3.99.3, IC₅₀ = 0.4 μ M) rather than COX-2. In fact, arzanol could block COX-2/mPGES-1-mediated PGE₂ biosynthesis in lipopolysaccharide-stimulated human monocytes and human whole blood, but not the concomitant COX-2-derived biosynthesis of thromboxane B₂ or of 6-keto PGF_{1 α} , 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 PGE₂ in the pleural exudates. Taken together, our data show that arzanol potently inhibits the biosynthesis of pro-inflammatory lipid mediators like PGE₂ *in vitro* and *in vivo*, providing a mechanistic rationale for the anti-inflammatory activity of *Helichrysum italicum*, and a rationale for further pre-clinical evaluation of this novel anti-inflammatory lead.

Key words: 5-lipoxygenase, leukotriene, prostaglandin, inflammation, phloroglucinol

Abbreviations used: 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 E₂ 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 CaCl₂; TBH, *tert*-butyl hydroperoxide.

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 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)E₂ without affecting homeostatic PGE₂ and PGI₂ 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 constituent 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 forties and fifties. However, his findings were published in little known Journals, and, after his death, were largely overlooked [8]. Arzanol showed antioxidant 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 (NF κ B) activation and HIV-1 replication in T cells (IC₅₀ ~ 5 μ g/ml and 5 μ M, respectively) as well as the release of pro-inflammatory mediators such as interleukin (IL)-1 β , IL-6, IL-8, tumor necrosis factor (TNF) α , and PGE₂ 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 PGE₂ synthase (mPGES)-1 (EC 5.3.99.3), COX-1, and 5-LO in various test systems with IC₅₀ values between 0.4 to 9 μ M, and significantly reduces the inflammatory response and the PGE₂ 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)-1*H*-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); PGH₂, Larodan (Malmö, Sweden); 11 β -PGE₂, PGB₁, MK-886 (3-[1-(4-chlorobenzyl)-3-*t*-butyl-thio-5-isopropylindol-2-yl]-2,2-dimethylpropanoic acid), 6-keto PGF_{1 α} , Cayman Chemical (Ann Arbor, MI); Ultima GoldTM XR, Perkin Elmer (Boston, MA); [³H]-PGE₂ was from PerkinElmer Life Sciences (Milan, Italy) and PGE₂ 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% CO₂ incubator. After 3 days, confluent cells were detached using 1 × trypsin/EDTA solution and reseeded at 2 × 10⁶ cells in 20 ml medium in 175 cm² 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 CaCl₂. 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 CaCl₂ (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 × 10⁷ 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

1 molecule by flow cytometry (BD FACS Calibur, Rockville, MD). Monocytes were finally
2 resuspended in ice-cold PBS plus 1 mg/ml glucose or in PGC buffer.
3

4
5
6
7 Cell viability was assessed by trypan blue staining and light microscopy as well as by 3-(4,5-
8 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, A549 cells ($4 \times$
9 10^5 , plated into a 75 cm³ cell culture flask and grown for 72 h) or human PBMC (10^6 cells/ml)
10 were incubated at 37 °C and 5% CO₂. Then, arzanol was added and the incubation was
11 continued for 24 h before cell viability was determined. Treatment with arzanol (10 or 30 µM)
12 for 24 h did not significantly reduce cell viability in both cell cultures, excluding acute
13 cytotoxic effects in the cellular assays used in this study. However, for A549 cells, incubation
14 with 50 µM arzanol led to an increase in the ratio of dead cells to total number of cells (80%
15 dead cells), whereas for the PBMC population no significant reduction of cell viability was
16 evident at 50 µM arzanol (not shown).
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32

33 **2.3. Animals**

34
35
36
37
38 Male adult Wistar Han rats (200-220 g, Harlan, Milan, Italy) were housed in a controlled
39 environment and provided with standard rodent chow and water. Animal care complied with
40 Italian regulations on protection of animals used for experimental and other scientific purpose
41 (Ministerial Decree 116192) as well as with the European Economic Community regulations
42 (Official Journal of E.C. L 358/1 12/18/1986).
43
44
45
46
47
48
49
50
51
52

53 **2.4. DPPH Assay**

54
55
56
57
58 The radical scavenger capability was assessed by measuring the reduction of the stable free
59 radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). Briefly, an ethanol solution of a sample at
60
61
62
63
64
65

various concentrations (100 μ l) was mixed with an acetate-buffered (pH 5-6.5) DPPH solution (final concentration 50 μ M). 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 $\times$ 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 μ g/ml), 1 mM phenylmethanesulphonyl fluoride, and lysozyme (500 μ g/ml), homogenized by sonication (3 $\times$ 15 sec), and centrifuged at 40 000 $\times$ g for 20 min at 4 °C. The 40 000 $\times$ 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 sec at 37 °C, and 2 mM CaCl_2 plus 20 μ M 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 μ M ionophore A23187 plus 20 μ M AA. After 10 min at 37 °C, the reaction was stopped with 1 ml of methanol and 30 μ l of 1 N HCl, and then, 200 ng PGB₁ and 500 μ l PBS were added. Formed 5-LO metabolites were extracted and analyzed by HPLC as described [16]. 5-LO products include LTB₄ and its all-trans isomers, and 5(*S*)-hydro(pero)xy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid. Cysteinyl-LTs C₄, D₄, and E₄ were not detected, and oxidation products of LTB₄ were not determined.

For stimulation with LPS/fMLP, freshly isolated neutrophils (2×10^7 /ml PGC buffer) were incubated with LPS (1 μ g/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 μ M fMLP. After 5 min samples were placed on ice for 2 min, centrifuged (600 $\times$ g, 10 min), and LTB₄ was determined in the supernatant using a LTB₄ 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 $\times$ 5 sec at 4 °C) of freshly isolated platelets (10^8 /ml PBS containing 1 mM EDTA). Monocytes were stimulated with LPS (10 μ g/ml) for 20 h to induce COX-2 expression and cell homogenates were prepared by sonication (3 $\times$ 5 sec at 4 °C; 4×10^6 /ml PBS containing 1 mM EDTA). After

addition of 5 mM glutathione and 5 μ M 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 μ M 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 μ M 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 $\text{PGF}_{1\alpha}$ formation in intact A549 cells

The formation of 6-keto $\text{PGF}_{1\alpha}$ 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-1 β (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 $\text{PGF}_{1\alpha}$ formation was initiated by addition of AA (1 μ M). After 30 min at 37 °C, the reaction was stopped by cooling on ice. Cells were centrifuged (300 $\times$ g, 5 min, 4 °C), and the amount of released 6-keto $\text{PGF}_{1\alpha}$ was assessed by the High Sensitivity EIA Kit (Assay Designs, Ann Arbor, MI) according to the manufacturer's protocol.

2.11. Determination of PGE₂ formation in intact human monocytes

Formation of PGE₂ in intact cells was determined in monocytes according to Karlsson et al. [19]. In brief, freshly isolated monocytes were cultured in twelve-well plates (density 10⁶/ml per well) in RPMI medium containing 0.5% FCS and pre-treated with LPS (1 µg/ml) for 20 h. Cells were washed 3 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. PGE₂ formation was initiated by addition of AA (1 µM). After 30 min, the amount of PGE₂ in the supernatant was measured using a PGE₂ High Sensitivity EIA Kit (Assay Designs, Ann Arbor, MI) according to the manufacturer's protocol.

2.12. SDS-PAGE and Western blot

Monocytes (5 × 10⁶ 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 PGE₂, 6-keto PGF_{1α}, and TXB₂ formation in human whole blood

Peripheral blood from healthy adult volunteers, who had not received any medication for at least two weeks under informed consent, was obtained by venepuncture and collected in syringes containing heparin (20 U/ml). Aliquots of whole blood (PGE₂: 0.8 ml, 6-keto PGF_{1α}: 0.4 ml) were mixed with CV4151 (1 μM) and with aspirin (50 μM) for determination of PGE₂ and with CV4151 (1 μM) for analysis of 6-keto PGF_{1α}, respectively. For determination of TXB₂, 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_{1α} and TXB₂ were quantified in the supernatant using High Sensitivity EIA Kits (Assay Designs, Ann Arbor, MI), respectively, according to the manufacturer's protocols. PGE₂ 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 PGE₂. The PGE₂ 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 PGE₂ contents were quantified using a PGE₂ 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 PGE₂ 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-1 β for 48 h at 37 °C and 5% CO₂. After sonication, the homogenate was subjected to differential centrifugation at 10 000 $\times$ g for 10 min and 174 000 $\times$ 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 phenylmethanesulphonyl fluoride, 60 μ g/ml soybean trypsin inhibitor, 1 μ g/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 μ l total volume) was initiated by addition of PGH₂ (20 μ M, final concentration, unless stated otherwise). After 1 min at 4 °C, the reaction was terminated using stop solution (100 μ l; 40 mM FeCl₂, 80 mM citric acid, and 10 μ M of 11 β -PGE₂ as internal standard). PGE₂ 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 O₂, 0.5 l/min, N₂O 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 CO₂. 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 PGE₂, LTB₄ and 6-keto PGF_{1α} in the supernatant of centrifuged exudate (800×g for 10 min) were assayed by radioimmunoassay (PGE₂) and enzyme immunoassay (LTB₄, 6-keto PGF_{1α}), 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. IC₅₀ 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 antioxidant 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 μ M, **Fig. 1B**). Based on the antioxidant 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 $IC_{50} = 3.1$ μ M. Wash-out experiments demonstrated that this effect was reversible, as incubation of 5-LO with 10 μ M arzanol and subsequent dilution to 1 μ M (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 μ M) as well as for cells primed with LPS (1 μ g/ml, 30 min) and then stimulated with 100 nM fMLP ($IC_{50} = 2.9$ and 8.1 μ M, respectively, **Fig. 1E**). An interference with AA supply, e.g., by inhibition of cPLA₂ can be excluded, because for A23187-activated neutrophils exogenous AA was provided, and thus endogenous substrate supply was circumvented. Also, arzanol up to 10 μ M failed to inhibit isolated recombinant human cPLA₂ whereas the pyrrolidine cPLA_{2 α} inhibitor (5 μ M) was active (not shown). The 5-LO inhibitor BWA4C (0.3 μ M), 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 TXB₂ after addition of the COX substrate AA (5 μ M). The formation of both 12-HHT and TXB₂ was concentration-dependently inhibited by arzanol with IC₅₀ = 2.3 and 2.9 μ M, 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 (IC₅₀ approx. 20 μ M, 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 (IC₅₀ = 17.5 μ M, **Fig. 2B**).

For analysis of modulation of COX-2 activity, human lung epithelial A549 cells were treated with IL-1 β to induce COX-2 and to form the stable PGI₂ degradation product 6-keto PGF_{1 α} (as biomarker for PGI₂ formation) from exogenous AA (1 μ M) [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 α} formation, with an apparent IC₅₀ $\geq$ 30 μ M (**Fig. 3A**). Higher concentrations of arzanol were not tested because at 50 μ M, but not at 30 μ M, arzanol significantly impaired cell viability (by 80% within 24 h, assessed by MTT assay, not shown). Along these lines, arzanol up to 30 μ M failed to substantially inhibit COX-2 in a cell-free assay where human monocytes were used

as enzyme source; indomethacin (10 μ M) and the COX-2 selective celecoxib (5 μ M) clearly blocked COX-2 activity (**Fig. 3B**).

In addition, we investigated the effects of arzanol on cellular COX-2-derived PGE₂ formation in intact human monocytes derived from peripheral blood that were stimulated with LPS for 24 h and subsequently PGE₂ was assessed by ELISA [19]. Monocyte-based PGE₂ 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 [³H]AA [3]. Thus, IL-1 β -treated A549 cells might produce substantial amounts of other prostanoids (e.g., 6-keto PGF_{1 α}) and related products that seemingly interfere with commercially available PGE₂ ELISA detection systems, see also [18]. The COX inhibitor indomethacin and the mPGES-1 inhibitor MD52 were used as controls. Arzanol significantly inhibited PGE₂ formation already at 3 μ M and the IC₅₀ was determined at 9 μ M (**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 PGE₂ formation in human whole blood stimulated with LPS for 5 h, starting at 3 μ M, and at 30 μ M arzanol PGE₂ synthesis was repressed by about 50% (**Fig. 4A**). In parallel, the accompanied COX-2-mediated biosynthesis of TXB₂ (**Fig. 4B**) and the COX-2-derived 6-keto PGF_{1 α} 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 PGE₂ synthesis in LPS-treated monocytes and whole blood, but hardly affecting 6-keto PGF_{1 α} 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 PGE₂ which is clearly suppressed.

3.3. Effects of arzanol on mPGES-1

The suppression of PGE₂ 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-1 β -stimulated A549 cells, used as source of mPGES-1 [21], were pre-incubated with test compounds for 15 min, and then, PGE₂ formation was initiated by addition of 20 μ M PGH₂. The mPGES-1 inhibitor MK-886 (used as control) blocked PGE₂ formation with an IC₅₀ of 2.1 μ M (data not shown). Arzanol concentration-dependently inhibited PGE₂ formation with an IC₅₀ = 0.4 μ M (**Fig. 5A**) being superior over MK-886. Alteration of PGH₂ to lower (1 μ M) or higher (50 μ M) 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 PGE₂ synthesis in a reversible manner, wash-out experiments were carried out. Arzanol failed to efficiently block PGE₂ synthesis at 0.1 μ M, whereas PGE₂ formation was efficiently inhibited at 1 μ M (**Fig. 5C**). However, 10-fold dilution of the samples containing 1 μ M arzanol restored mPGES-1 activity implying a reversible mode of inhibition. It appeared possible that arzanol could directly act on PGH₂ and thus inhibit non-enzymatic PGE₂ formation independent of mPGES-1. However, when the microsomal preparations of A549 cells were heat-inactivated (15 min/65 °C) the non-enzymatic PGE₂ formation was not affected by arzanol up to 10 μ M (not shown).

3.4. Arzanol suppresses carrageenan-induced pleurisy in rats and inhibits eicosanoid biosynthesis *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 hours, 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 PGE₂ levels (47% inhibition), whereas indomethacin almost completely suppressed PGE₂ (95%) as well as 6-keto PGF_{1α} (94%) levels as expected. In agreement with the moderate inhibition of COX-2-derived 6-keto PGF_{1α} formation in A549 cells or human whole blood (see above), arzanol also slightly reduced the levels of 6-keto PGF_{1α} (27% inhibition). On the other hand, indomethacin failed to significantly reduce LTB₄ 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 antioxidant 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 NF κ B pathway [8, 12]. We now report that arzanol potently inhibits the biosynthesis of PGE₂, TXB₂, 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 TXA₂ and COX-2-derived PGE₂, and the minor suppression of beneficial prostanoids (e.g., PGI₂, the instable precursor of 6-keto PGF_{1 α}) 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 NF κ B activation and release of pro-inflammatory cytokines (i.e., IL-1 β , IL-6, IL-8 and TNF α) [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 ($IC_{50} = 3.1 \mu M$) is equipotent to caffeic acid ($IC_{50} = 3.7 \mu M$ [31]) and even more potent than nordihydroguaiaretic acid (NDGA, $IC_{50} = 28 \mu M$ [32]), magnolol ($IC_{50} = 15 - 25 \mu M$ [33]) or the structurally related myrtucommulone ($IC_{50} = 5 \mu M$ [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 cPLA₂ 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 μ M 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 TXB₂ 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 ($IC_{50} = 2.3 - 2.9 \mu$ M) is equipotent or even more potent than ibuprofen ($IC_{50} = 3 - 70 \mu$ M [38, 39]) or aspirin ($IC_{50} = 3.2 \mu$ M [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 PGE₂ synthesis and our data suggest that the selective suppression of PGE₂ 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 ($IC_{50} \geq 30 \mu$ M) under conditions where COX-1 was potently blocked. In IL-1 β -treated A549 cells expressing solely COX-2, arzanol (30 μ M) caused only modest inhibition of 6-keto PGF_{1 α} formation. However, arzanol (3 μ M) efficiently inhibited PGE₂ 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.

1 Divergent effects of arzanol on COX-2-derived prostanoid formation were also apparent when
2 the compound was analyzed in the human whole blood assay. Neither TXB₂ synthesis nor
3
4 formation of 6-keto PGF_{1α} were significantly reduced up to 30 μM arzanol in this assay,
5
6 whereas under the same conditions arzanol suppressed PGE₂ synthesis at relatively low
7
8 concentrations (3 μM) but reached a plateau at higher concentrations (30 μM) where about
9
10 50% PGE₂ still remained. Also the potent mPGES-1 inhibitor MD52 suppressed PGE₂
11
12 formation only by 50%, and failed to block TXB₂ and 6-keto-PGF_{1α}, supporting mPGES-1 as
13
14 the respective target of arzanol. Presumably, other PGES isoforms unaffected by MD52 or
15
16 arzanol contribute to the basal PGE₂ generation in whole blood. On the other hand, the PGE₂
17
18 ELISA detection systems may recognize related (non-prostanoid) cross-reactive substances,
19
20 supported by the incomplete suppression of PGE₂ formation by indomethacin in whole blood
21
22 while TXB₂ and 6-keto-PGF_{1α} synthesis is effectively inhibited.
23
24
25
26
27
28
29
30

31 Arzanol reduced the inflammatory reaction in an *in vivo* model of acute inflammation, the
32
33 carrageenan-induced rat pleurisy, with activity almost comparable to that of indomethacin.
34
35 PGE₂ plays a central role in the early phase of carrageenan-induced pleurisy [42], and COX-
36
37 inhibitors prevent the inflammatory response. Accordingly, lowering PGE₂ by inhibition of
38
39 mPGES-1 may contribute to the anti-inflammatory properties of arzanol. As observed in the
40
41 whole blood assay, arzanol was less potent than indomethacin in the reduction of PGE₂ levels,
42
43 but still efficiently suppressed exudate formation and infiltration of inflammatory cells. In
44
45 contrast to indomethacin, arzanol also reduced the levels of LTB₄ in the pleural exudates.
46
47 Since 5-LO-derived metabolites cooperate with PGE₂ in the induction of pleurisy [43],
48
49 inhibition of 5-LO may contribute to the anti-inflammatory effect of arzanol. Indeed,
50
51 synergistic anti-inflammatory effects of so-called dual 5-LO/COX have been observed [25,
52
53 44]. The concomitant blockade of LTB₄ formation is seemingly also responsible for the lack
54
55 of gastric toxicity (the most troublesome side effect of NSAIDs) observed with 5-LO/COX
56
57
58
59
60
61
62
63
64
65

1 dual inhibitors [44]. Future studies using animal models of gastric toxicity will reveal whether
2 arzanol might exert its anti-inflammatory activity without gastric side effects. Taken together,
3
4 our data show that arzanol potently inhibits the biosynthesis of pro-inflammatory lipid
5
6 mediators like PGE₂ *in vitro* and *in vivo*, providing a mechanistic rationale for the anti-
7
8 inflammatory activity of *H. italicum*, and a rationale for further pre-clinical evaluation of this
9
10 novel anti-inflammatory lead.
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Acknowledgments

The authors thank Bianca Jazzar and Daniela Mueller for expert technical assistance and we thank Aureliasan GmbH (Tuebingen, Germany) for financial support.

Conflict of Interest Statement: None declared.

References

- [1] Funk CD. Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science* 2001;294:1871-5.
- [2] Rainsford KD. Anti-inflammatory drugs in the 21st century. *Subcell Biochem* 2007;42:3-27.
- [3] Koeberle A, Werz O. Inhibitors of the microsomal prostaglandin E(2) synthase-1 as alternative to non steroidal anti-inflammatory drugs (NSAIDs)--a critical review. *Curr Med Chem* 2009;16:4274-96.
- [4] Radmark O, Samuelsson B. Microsomal prostaglandin E synthase-1 and 5-lipoxygenase: potential drug targets in cancer. *J Intern Med.*; 2010; 268:5-14.
- [5] Werz O. Inhibition of 5-lipoxygenase product synthesis by natural compounds of plant origin. *Planta Med* 2007;73:1331-57.
- [6] Jachak SM. Cyclooxygenase inhibitory natural products: current status. *Curr Med Chem* 2006;13:659-78.
- [7] Sala A, Recio M, Giner RM, Manez S, Tournier H, Schinella G, et al. Anti-inflammatory and antioxidant properties of *Helichrysum italicum*. *J Pharm Pharmacol* 2002;54:365-71.
- [8] Appendino G, Ottino M, Marquez N, Bianchi F, Giana A, Ballero M, et al. Arzanol, an anti-inflammatory and anti-HIV-1 phloroglucinol alpha-Pyrone from *Helichrysum italicum* ssp. *microphyllum*. *J Nat Prod* 2007;70:608-12.
- [9] Nostro A, Bisignano G, Angela Cannatelli M, Crisafi G, Paola Germano M, Alonzo V. Effects of *Helichrysum italicum* extract on growth and enzymatic activity of *Staphylococcus aureus*. *Int J Antimicrob Agents* 2001;17:517-20.
- [10] Angioni A, Barra A, Arlorio M, Coisson JD, Russo MT, Pirisi FM, et al. Chemical composition, plant genetic differences, and antifungal activity of the essential oil of

- 1 Helichrysum italicum G. Don ssp. microphyllum (Willd) Nym. J Agric Food Chem
- 2 2003;51:1030-4.
- 3
- 4 [11] Nostro A, Cannatelli MA, Marino A, Picerno I, Pizzimenti FC, Scoglio ME, et al.
- 5
- 6 Evaluation of antiherpesvirus-1 and genotoxic activities of Helichrysum italicum
- 7
- 8 extract. New Microbiol 2003;26:125-8.
- 9
- 10
- 11 [12] Rosa A, Deiana M, Atzeri A, Corona G, Incani A, Melis MP, et al. Evaluation of the
- 12
- 13 antioxidant and cytotoxic activity of arzanol, a prenylated alpha-pyrone-
- 14
- 15 phloroglucinol etherodimer from Helichrysum italicum subsp.microphyllum. Chem
- 16
- 17 Biol Interact 2007;165:117-26.
- 18
- 19
- 20
- 21 [13] Cote B, Boulet L, Brideau C, Claveau D, Ethier D, Frenette R, et al. Substituted
- 22
- 23 phenanthrene imidazoles as potent, selective, and orally active mPGES-1 inhibitors.
- 24
- 25 Bioorg Med Chem Lett 2007;17:6816-20.
- 26
- 27
- 28
- 29 [14] Pergola C, Dodt G, Rossi A, Neunhoeffler E, Lawrenz B, Northoff H, et al. ERK-
- 30
- 31 mediated regulation of leukotriene biosynthesis by androgens: a molecular basis for
- 32
- 33 gender differences in inflammation and asthma. Proc Natl Acad Sci U S A
- 34
- 35 2008;105:19881-6.
- 36
- 37
- 38
- 39 [15] Fischer L, Szellas D, Radmark O, Steinhilber D, Werz O. Phosphorylation- and
- 40
- 41 stimulus-dependent inhibition of cellular 5-lipoxygenase activity by nonredox-type
- 42
- 43 inhibitors. FASEB Journal 2003;17:949-51.
- 44
- 45
- 46 [16] Werz O, Burkert E, Samuelsson B, Radmark O, Steinhilber D. Activation of 5-
- 47
- 48 lipoxygenase by cell stress is calcium independent in human polymorphonuclear
- 49
- 50 leukocytes. Blood 2002;99:1044-52.
- 51
- 52
- 53 [17] Albert D, Zundorf I, Dingermann T, Muller WE, Steinhilber D, Werz O. Hyperforin is
- 54
- 55 a dual inhibitor of cyclooxygenase-1 and 5-lipoxygenase. Biochem Pharmacol
- 56
- 57 2002;64:1767-75.
- 58
- 59
- 60
- 61
- 62
- 63
- 64
- 65

- [18] Koeberle A, Siemoneit U, Buehring U, Northoff H, Laufer S, Albrecht W, et al. Licofelone suppresses prostaglandin E2 formation by interference with the inducible microsomal prostaglandin E2 synthase-1. *J Pharmacol Exp Ther* 2008;326:975-82.
- [19] Karlsson S, Nanberg E, Fjaeraa C, Wijkander J. Ellagic acid inhibits lipopolysaccharide-induced expression of enzymes involved in the synthesis of prostaglandin E2 in human monocytes. *Br J Nutr* 2009;1-8.
- [20] Thoren S, Jakobsson PJ. Coordinate up- and down-regulation of glutathione-dependent prostaglandin E synthase and cyclooxygenase-2 in A549 cells. Inhibition by NS-398 and leukotriene C4. *Eur J Biochem* 2000;267:6428-34.
- [21] Jakobsson PJ, Thoren S, Morgenstern R, Samuelsson B. Identification of human prostaglandin E synthase: a microsomal, glutathione-dependent, inducible enzyme, constituting a potential novel drug target. *Proc Natl Acad Sci U S A* 1999;96:7220-5.
- [22] Feisst C, Pergola C, Rakonjac M, Rossi A, Koeberle A, Dodt G, et al. Hyperforin is a novel type of 5-lipoxygenase inhibitor with high efficacy in vivo. *Cell Mol Life Sci* 2009;66:2759-71.
- [23] Rossi A, Di Paola R, Mazzon E, Genovese T, Caminiti R, Bramanti P, et al. Myrtucommulone from *Myrtus communis* exhibits potent anti-inflammatory effectiveness in vivo. *J Pharmacol Exp Ther* 2009;329:76-86.
- [24] Werz O, Steinhilber D. Therapeutic options for 5-lipoxygenase inhibitors. *Pharmacol Ther* 2006;112:701-18.
- [25] Kulkarni SK, Singh VP. Licofelone: the answer to unmet needs in osteoarthritis therapy? *Curr Rheumatol Rep* 2008;10:43-8.
- [26] Fischer L, Hornig M, Pergola C, Meindl N, Franke L, Tanrikulu Y, et al. The molecular mechanism of the inhibition by licofelone of the biosynthesis of 5-lipoxygenase products. *Br J Pharmacol* 2007;152:471-80.

- [27] Koeberle A, Rossi A, Zettl H, Pergola C, Dehm F, Bauer J, et al. The molecular pharmacology and in vivo activity of 2-(4-chloro-6-(2,3-dimethylphenylamino)pyrimidin-2-ylthio)octanoic acid (YS121), a dual inhibitor of microsomal prostaglandin E2 synthase-1 and 5-lipoxygenase. *J Pharmacol Exp Ther* 2010;332:840-8.
- [28] Koeberle A, Pollastro F, Northoff H, Werz O. Myrtucommulone, a natural acylphloroglucinol, inhibits microsomal prostaglandin E(2) synthase-1. *Br J Pharmacol* 2009;156:952-61.
- [29] Smith WL, DeWitt DL, Garavito RM. Cyclooxygenases: structural, cellular, and molecular biology. *Annu Rev Biochem* 2000;69:145-82.
- [30] Radmark O, Werz O, Steinhilber D, Samuelsson B. 5-Lipoxygenase: regulation of expression and enzyme activity. *Trends Biochem Sci* 2007;32:332-41.
- [31] Koshihara Y, Neichi T, Murota S, Lao A, Fujimoto Y, Tatsuno T. Selective inhibition of 5-lipoxygenase by natural compounds isolated from Chinese plants, *Artemisia rubripes* Nakai. *FEBS Lett* 1983;158:41-4.
- [32] Laughton MJ, Evans PJ, Moroney MA, Hoult JR, Halliwell B. Inhibition of mammalian 5-lipoxygenase and cyclo-oxygenase by flavonoids and phenolic dietary additives. Relationship to antioxidant activity and to iron ion-reducing ability. *Biochem Pharmacol* 1991;42:1673-81.
- [33] Hsu MF, Lu MC, Tsao LT, Kuan YH, Chen CC, Wang JP. Mechanisms of the influence of magnolol on eicosanoid metabolism in neutrophils. *Biochem Pharmacol*, 2004. p. 831-40.
- [34] Feisst C, Franke L, Appendino G, Werz O. Identification of molecular targets of the oligomeric nonprenylated acylphloroglucinols from *Myrtus communis* and their

- implication as anti-inflammatory compounds. *J Pharmacol Exp Ther* 2005;315:389-96.
- [35] Bonventre JV, Huang Z, Taheri MR, O'Leary E, Li E, Moskowitz MA, et al. Reduced fertility and postischaemic brain injury in mice deficient in cytosolic phospholipase A2. *Nature* 1997;390:622-5.
- [36] Kurumbail RG, Stevens AM, Gierse JK, McDonald JJ, Stegeman RA, Pak JY, et al. Structural basis for selective inhibition of cyclooxygenase-2 by anti-inflammatory agents. *Nature* 1996;384:644-8.
- [37] Marnett LJ, Rowlinson SW, Goodwin DC, Kalgutkar AS, Lanzo CA. Arachidonic acid oxygenation by COX-1 and COX-2. Mechanisms of catalysis and inhibition. *J Biol Chem* 1999;274:22903-6.
- [38] Kawai S, Nishida S, Kato M, Furumaya Y, Okamoto R, Koshino T, et al. Comparison of cyclooxygenase-1 and -2 inhibitory activities of various nonsteroidal anti-inflammatory drugs using human platelets and synovial cells. *Eur J Pharmacol* 1998;347:87-94.
- [39] Boneberg EM, Zou MH, Ullrich V. Inhibition of cyclooxygenase-1 and -2 by R(-)- and S(+)-ibuprofen. *J Clin Pharmacol* 1996;36:16S-9S.
- [40] McQuay HJ, Moore RA. Dose-response in direct comparisons of different doses of aspirin, ibuprofen and paracetamol (acetaminophen) in analgesic studies. *Br J Clin Pharmacol* 2007;63:271-8.
- [41] Kato M, Nishida S, Kitasato H, Sakata N, Kawai S. Cyclooxygenase-1 and cyclooxygenase-2 selectivity of non-steroidal anti-inflammatory drugs: investigation using human peripheral monocytes. *J Pharm Pharmacol* 2001;53:1679-85.
- [42] Kawamura M, Hatanaka K, Saito M, Ogino M, Ono T, Ogino K, et al. Are the anti-inflammatory effects of dexamethasone responsible for inhibition of the induction of

enzymes involved in prostanoid formation in rat carrageenin-induced pleurisy? Eur J
Pharmacol 2000;400:127-35.

- [43] Cuzzocrea S, Rossi A, Serraino I, Mazzon E, Di Paola R, Dugo L, et al. 5-Lipoxygenase knockout mice exhibit a resistance to pleurisy and lung injury caused by carrageenan. J Leukoc Biol 2003;73:739-46.
- [44] Celotti F, Laufer S. Anti-inflammatory drugs: new multitarget compounds to face an old problem. The dual inhibition concept. Pharmacol Res 2001;43:429-36.

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

Thirty min 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 $\pm$ SEM * p <0.05; *** p <0.01 vs vehicle

treatment	exudate volume (ml)	inflammatory cells $\times 10^6$	PGE ₂ (ng/rat)	LTB ₄ (ng/rat)	6-keto PGF _{1α} (ng/rat)
vehicle	0.41 $\pm$ 0.019	46.5 $\pm$ 6.0	4.26 $\pm$ 0.67	1.22 $\pm$ 0.26	10.72 $\pm$ 1.43
arzanol (3.6 mg/kg)	0.17 $\pm$ 0.05*** (59%)	24.0 $\pm$ 5.9* (48%)	2.27 $\pm$ 0.35* (47%)	0.84 $\pm$ 0.21 (31%)	7.79 $\pm$ 0.72 (27%)
indomethacin (5 mg/kg)	0.10 $\pm$ 0.03*** (76%)	16.28 $\pm$ 2.30*** (65%)	0.21 $\pm$ 0.024*** (95%)	1.06 $\pm$ 0.20 (13%)	0.64 $\pm$ 0.09 (94%)

Figure Legends

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 sec at 37 °C, and 5-LO product formation was started by addition of 2 mM CaCl₂ 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 sec at 37 °C, and 5-LO product formation was started by addition of 2 mM CaCl₂ 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 × 10⁶ 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.

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 ($5 \mu\text{M}$). 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 $5 \mu\text{M}$ haemoglobin and pre-incubated with the test compounds or vehicle (veh, DMSO) for 4 min at RT and 1 min at 37°C . Then, $5 \mu\text{M}$ 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 ($10 \mu\text{M}$).

Fig. 3. Effects of arzanol on COX-2 activity.

(A) 6-Keto $\text{PGF}_{1\alpha}$ formation as parameter of COX-2 activity. IL- 1β -stimulated A549 cells (1×10^6 /ml) were pre-incubated with arzanol, indomethacin (Indo, $10 \mu\text{M}$) or vehicle (veh, DMSO) as indicated for 15 min at RT prior to addition of $1 \mu\text{M}$ AA. After 30 min at 37°C the amount of 6-keto $\text{PGF}_{1\alpha}$ was assessed by ELISA as described. The 100% value corresponds to an average of 1.2 ng/ml 6-keto $\text{PGF}_{1\alpha}$. (B) COX-2 activity in monocyte homogenates. Monocytes were stimulated with LPS ($10 \mu\text{g}/\text{ml}$) for 20 h, cell homogenates were prepared, 5 mM glutathione and $5 \mu\text{M}$ haemoglobin were added, and pre-incubated with arzanol, indomethacin (Indo, $10 \mu\text{M}$), celecoxib (Cele, $5 \mu\text{M}$) or vehicle (veh, DMSO) for 4 min at RT and 1 min at 37°C . Then, $5 \mu\text{M}$ 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 ($1 \mu\text{g}/\text{ml}$) for 20 h, cells were washed, and pre-incubated with arzanol, indomethacin

(Indo, 10 μ M), MD52 (2 μ M) or vehicle (veh, DMSO) for 15 min. PGE₂ formation was initiated by addition of AA (1 μ M), and after 30 min, PGE₂ 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 μ g/ml LPS together with arzanol, dexamethasone (1 μ M) 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) PGE₂ formation. Heparinized human blood, treated with 1 μ M thromboxane synthase inhibitor and 50 μ M aspirin, was pre-incubated with arzanol, indomethacin (Indo, 50 μ M), MD52 (6 μ M) or vehicle (veh, DMSO) for 5 min at RT, and then, PGE₂ formation was induced by addition of 10 μ g/ml LPS. After 5 h at 37 °C, PGE₂ was extracted from plasma and analyzed as described, The 100% value corresponds to an average of 1.9 ng/ml PGE₂, and 0.42 ng/ml PGE₂ were detected in unstimulated blood. **(B)** TXB₂ formation. Heparinized blood was pre-incubated with arzanol, indomethacin (Indo, 50 μ M) or vehicle (veh, DMSO, w/o) for 5 min, then 10 μ g/ml LPS were added and TXB₂ was analyzed after 5 h at 37 °C in the plasma by ELISA. The 100% value corresponds to an average of 58 ng/ml TXB₂. **(C)** 6-Keto PGF_{1 α} formation. Heparinized human blood, treated with 1 μ M thromboxane synthase inhibitor, was pre-incubated with arzanol, indomethacin (Indo, 50 μ M), MD52 (6 μ M) or vehicle (veh, DMSO) for 5 min at RT, prior to addition of 10 μ g/ml LPS. After 5 min at 37 °C, 6-keto PGF_{1 α} was directly analyzed in plasma by ELISA. The 100% value corresponds to an average of and 14.5 ng/ml 6-keto PGF_{1 α} , and 2.3 ng/ml 6-keto PGF_{1 α} were detected in

unstimulated blood. Data are given as mean $\pm$ 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-1 β -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 μ M PGH₂. After 1 min at 4 °C, the reaction was terminated using a stop solution containing FeCl₂ and 11 β -PGE₂ (1 nmol) as internal standard. The 100% value corresponds to an average of 0.8 nmol PGE₂. (B) The potency of arzanol for mPGES-1 inhibition was compared at 1, 20, and 50 μ M PGH₂ as substrate. The amount of PGE₂ was quantified for 1 μ M PGH₂ by use of a PGE₂ High Sensitivity EIA Kit according to the manufacturer's protocol; for 20 and 50 μ M PGH₂, the PGE₂ was quantified by HPLC. The 100% values correspond to an average of 70 ng/ml (ELISA), or 1 and 5 nmol (HPLC) PGE₂ for 1, 20, and 50 μ M PGH₂, respectively. (C) Reversibility of mPGES-1 inhibition by arzanol. Microsomal preparations of IL-1 β -stimulated A549 cells were pre-incubated with 1 μ M inhibitor for 15 min at 4 °C. An aliquot was diluted 10-fold to obtain an inhibitor concentration of 0.1 μ M. For comparison, microsomal preparations were pre-incubated for 15 min with vehicle (veh, DMSO, w/o) or 0.1 μ M arzanol, and then, 20 μ M PGH₂ was added (no dilution). All samples were incubated for 1 min on ice, and PGE₂ formation was analyzed as described by HPLC. Data are given as mean $\pm$ S.E., $n = 3-4$, $*p < 0.05$ or $***p < 0.001$ vs. vehicle (0.1% DMSO) control, ANOVA + Tukey HSD *post-hoc* tests.

Figure 1

Fig. 1

Figure 2

Fig. 2

Figure 3

Fig. 3

Figure 4

Fig. 4

Figure 5

Fig. 5