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Identification of 5-lipoxygenase and microsomal prostaglandin E_2 synthase-1 as functional targets of the anti-inflammatory and anti-carcinogenic garcinol

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ABSTRACT

Garcinol (camboginol) from the fruit rind of Guttiferae species shows anti-carcinogenic and anti-inflammatory properties, but the underlying molecular mechanisms are unclear. Here we show that garcinol potently interferes with 5-lipoxygenase (EC 7.13.11.34) and microsomal prostaglandin (PG)E_2 synthase (mPGES)-1 (EC 5.3.99.3), enzymes that play pivotal roles in inflammation and tumorigenesis. In cell-free assays, garcinol inhibited the activity of purified 5-lipoxygenase and blocked the mPGES-1-mediated conversion of PGH_2 to PGE_2 with IC_{50} values of 0.1 and 0.3 μM, respectively. Garcinol suppressed 5-lipoxygenase product formation also in intact human neutrophils and reduced PGE_2 formation in interleukin-1β-stimulated A549 human lung carcinoma cells as well as in human whole blood stimulated by lipopolysaccharide. Moreover, garcinol interfered with isolated cyclooxygenase (COX)-1 (EC 1.14.99.1, IC_{50} = 12 μM) and with the formation of COX-1-derived 12(*S*)-hydroxy-5-cis-8,10-trans-heptadecatrienoic acid and thromboxane B_2 in human platelets. In contrast, neither Ca^{2+}-ionophore (A23187)-induced arachidonic acid release in neutrophils nor COX-2 activity in A549 cells or whole blood, measured as formation of 6-keto $PGF_{1\alpha}$, or isolated human recombinant COX-2 were significantly affected by garcinol (≤30 μM). Together, the high potency of garcinol to selectively suppress PGE_2 synthesis and 5-lipoxygenase product formation provides a molecular basis for the anti-inflammatory and anti-carcinogenic effects of garcinol and rationalizes its therapeutic use.

1. Introduction

Garcinol (Fig. 1A) is a polyprenylated acylphloroglucinole contained in the rind of Guttiferae (*Garcinia indica*, *Garcinia huillkensis* and *Garcinia cambogia*), named Kokum, which is used as culinary spice and garnish for curry. In India, Kokum is used in ayurvedic medicine for the treatment of inflammatory and infectious diseases [1]. In rats, garcinol protects against 4-nitroquinoline-1-oxide-induced tongue carcinogenesis [2] and azoxymethane-induced colon carcinogenesis [3]. Functional investigations have revealed anti-oxidative [4], anti-proliferative [5], pro-apoptotic [6], anti-invasive [7], and anti-inflammatory properties of garcinol [6,8,9]. Garcinol reduced the expression or activation of kinases involved in cell proliferation (i.e., focal adhesion kinase, extracellular signal-regulated kinase (ERK)-1/2, p38 mitogen-activated protein kinase [8,10]), blocked the expression of enzymes involved in the development and progression of cancer and inflammation (i.e., cyclooxygenase (COX)-2 (EC 1.14.99.1) and inducible nitric oxide synthase (iNOS)), and suppressed nuclear factor (NF)κB signalling [8,10]). However, molecular targets of garcinol are less defined, and thus far, only histone acetyltransferase p300 (IC_{50} = 7 μM) [11], P300/CBP-associated factor (IC_{50} = 5 μM) [11], and acetylcholine esterase [12] (IC_{50} = 0.7 μM) were identified.

Certain COX-1/2-derived prostanoids and 5-lipoxygenase (5-LO)-derived leukotrienes (LTs) initiate and maintain inflammatory and allergic reactions (for review see [13]), and pharmacological intervention with their biosynthesis (i.e., utilizing COX- and 5-LO (EC 7.13.11.34) inhibitors) is applied in therapy of respective diseases. COX-2 and/or 5-LO are also overexpressed in various cancer cells (e.g., prostate, breast, lung, and colon) [14–16], and pre-clinical studies indicate tumor-preventive effects of COX inhibition by non-steroidal anti-inflammatory drugs (NSAIDs) [17]. Moreover, pharmacological suppression of the 5-LO pathway, using 5-LO inhibitors or inhibitors of the 5-LO-activating protein (FLAP), causes apoptosis of various tumor cells [16,18]. Prostaglandin (PG)E_2 is the most prominent COX product in inflamma-

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Abbreviations: AA, arachidonic acid; COX, cyclooxygenase; cPL, cytosolic phospholipase; ERK, extracellular signal-regulated kinase; FLAP, 5-lipoxygenase-activating protein; 12-HHT, 12(*S*)-hydroxy-5-cis-8,10-trans-heptadecatrienoic acid; iNOS, inducible nitric oxide synthase; 5-LO, 5-lipoxygenase; LT, leukotriene; mPGES, microsomal prostaglandin E_2 synthase; NSAIDs, non-steroidal anti-inflammatory drugs; PG, prostaglandin.

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tion and tumorigenesis and is formed from PGH_2 by PGE_2 synthases. The microsomal PGE_2 synthase (mPGES)-1 (EC 5.3.99.3) is an inducible isoform functionally coupled to COX-2. Accumulating evidence suggests that mPGES-1 is responsible for massive PGE_2 formation associated with pathologies [19]. Therefore, mPGES-1 may represent an alternative drug target for treatment of inflammation and cancer, which is supported by data from mPGES-1 deficient mice and animal studies with selective mPGES-1 inhibitors [19–21].

Despite the anti-inflammatory and anti-tumorigenic potential of garcinol, its effects on the formation of eicosanoids were rarely analyzed. Hong et al. recently showed that garcinol modulates arachidonic acid (AA) metabolism in lipopolysaccharide-stimulated macrophages by blocking cytosolic phospholipase (cPL)A_2 (EC 3.1.1.4) activation due to inhibition of ERK1/2-mediated phosphorylation [10]. However, modulation of PG and/or LT biosynthesis by garcinol or effects on the catalytic activity of the key enzymes involved (e.g., COX-1/2, PG synthases, 5-LO) have not been addressed yet. Here, we demonstrate that garcinol inhibits prostanoid and LT formation in cellular and cell-free assays by preferential and potent interference with mPGES-1 and 5-LO.

2. Materials and methods

2.1. Reagents

Garcinol (Biomol, Hamburg, Germany) was dissolved in dimethyl sulphoxide (DMSO) and kept in the dark at −20 °C, and freezing/thawing cycles were kept to a minimum. The thromboxane synthase inhibitor CV4151 [22] and anti-6-keto $PGF_{1\alpha}$ antibody were generous gifts by Dr. S. Laufer (University of Tuebingen, Germany) and Dr. T. Dingermann (University of Frankfurt, Germany), respectively. Materials used: DMEM/High Glucose (4.5 g/l) medium, penicillin, streptomycin, trypsin/EDTA solution, PAA (Coelbe, Germany); PGH_2, Larodan (Malmö, Sweden); 11β-PGE_2, PGB_1, MK-886 (3-[1-(4-chlorobenzyl)-3-t-butylthio-5-isopropylindol-2-yl]-2,2-dimethylpropanoic acid), 6-keto $PGF_{1\alpha}$, human recombinant COX-2, ovine COX-1, Cayman Chemical (Ann Arbor, MI); [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid ([^{3}H]AA), BioTrend Chemicals GmbH (Cologne, Germany); Ultima Gold™ XR, PerkinElmer (Boston, MA). 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 (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. Cell viability was assessed by trypan blue staining and light microscopy. A549 cells (4×10^5) were plated in a 75 cm^3 cell culture flask and incubated at 37 °C and 5% CO_2 for 72 h. Then, garcinol was added and the incubation was continued for 5 or 24 h before cell viability was determined. Treatment with garcinol (10 or 30 μM) for 5 h did not significantly reduced cell viability, excluding acute cytotoxic effects in the cellular assays used in this study. However, prolonged incubation (24 h) with 10 or 30 μM garcinol led to a strong increase in the ratio of dead cells to total number of cells (50% and 71% dead cells, respectively).

Blood cells were freshly isolated from leukocyte concentrates obtained at the Blood Center of the University Hospital Tuebingen (Germany) as described [23]. 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, Coelbe, Germany). 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 [24]. Cells were finally resuspended in PBS pH 7.4 (PBS) containing 1 mg/ml glucose and 1 mM $CaCl_2$ (PGC buffer) (purity > 96–97%).

2.3. Release of arachidonic acid in neutrophils

Freshly isolated human neutrophils (3×10^7 ml^{-1} PGC buffer) were pre-incubated with the test compounds for 10 min prior to stimulation with Ca^{2+}-ionophore A23187 (2.5 μM). After 5 min at 37 °C, the reaction was stopped by addition of 1 ml of methanol. After adjusting pH 3 and addition of magarinic acid (11.1 nmol) as internal standard, released AA was separated by solid phase extraction (RP-18 material and elution with methanol) and coupled to 2,4-dimethoxyaniline hydrochloride (0.14 mg, 0.75 μmol) using *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide (1.46 mg, 9.4 μmol) as coupling reagent for 1 h at 37 °C. Derivatized AA was analyzed by RP-HPLC (gradient: 71% methanol/29% water to 100% methanol in 20 min, detection at 272 nm).

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

Freshly isolated platelets (10^8 ml^{-1} PBS containing 1 mM $CaCl_2$) were pre-incubated with the indicated agents for 5 min at room temperature. After addition of 5 μM AA and further incubation for 5 min at 37 °C, the COX-1-derived TxB_2 was quantified using a TxB_2 High Sensitivity EIA Kit (Assay Designs, Ann Arbor, MI). 12(*S*)-Hydroxy-5-cis-8,10-trans-heptadecatrienoic acid (12-HHT, non-enzymatically formed from PGH_2) was extracted and analyzed by HPLC as described [23].

2.5. Determination of PGE_2 and 6-keto $PGF_{1\alpha}$ formation in intact A549 cells

Cellular PGE_2 and 6-keto $PGF_{1\alpha}$ formation was determined according to [25]. 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 interleukin-1β (1 ng/ml) for 48 h. After trypsination, cells were washed with PBS twice. For determination of PGE_2, 4×10^6 cells per ml PBS containing $CaCl_2$ (1 mM) were pre-incubated with the indicated compounds at 37 °C for 10 min, and PGE_2 formation was started by addition of ionophore A23187 (2.5 μM), AA (1 μM), and [^{3}H]AA (18.4 kBq). The reaction was stopped after 15 min at 37 °C, and the samples were put on ice. After centrifugation (800 × *g*, 5 min, 4 °C), the supernatant was acidified (pH 3) by addition of citric acid (20 μl, 2 M), and the internal standard 11β-PGE_2 (2 nmol) was added. Radiolabeled PGE_2 was separated and analyzed by RP-18 solid phase extraction and HPLC analysis. The amount of 11β-PGE_2 was quantified by integration of the area under the eluted peaks. For quantification of radiolabeled PGE_2, fractions (0.5 ml) were collected and mixed with Ultima Gold™ XR (2 ml) for liquid scintillation counting in a LKB Wallac 1209 Rackbeta Liquid Scintillation Counter.

For determination of 6-keto $PGF_{1\alpha}$, 1×10^6 cells, resuspended in 1 ml PBS containing $CaCl_2$ (1 mM), were pre-incubated with the indicated compounds for 15 min at 37 °C, and 6-keto $PGF_{1\alpha}$ formation was initiated by addition of AA (30 μM). After 15 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 ELISA as described [25].

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

Inhibition of the activities of isolated ovine COX-1 and human COX-2 was performed as described [25]. Briefly, purified COX-1 (ovine, 50 units) or COX-2 (human recombinant, 20 units) were diluted in 1 ml reaction mixture containing 100 mM Tris buffer pH 8, 5 mM glutathione, 5 μM haemoglobin, and 100 μM EDTA at 4 °C and pre-incubated with the test compounds for 5 min. Samples were pre-warmed for 60 s at 37 °C, and AA (5 μM for COX-1, 2 μM for COX-2) was added to start the reaction. After 5 min at 37 °C, the COX-derived 12-HHT was extracted and then analyzed by HPLC as described [23].

2.7. 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 [25]. In brief, cells were incubated for 16 h at 37 °C and 5% CO_2, the medium was replaced, interleukin-1β (1 ng/ml) was added and cells were incubated for another 48 h. Cells were harvested, frozen in liquid nitrogen, and ice-cold homogenization buffer (0.1 M potassium phosphate buffer pH 7.4, 1 mM phenylmethanesulphonyl fluoride, 60 μg/ml soybean trypsin inhibitor, 1 μg/ml leupeptin, 2.5 mM glutathione, and 250 mM sucrose) was added. After 15 min, cells were sonicated on ice (3 × 20 s) and 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 and the total protein concentration was determined by Coomassie protein assay [26]. 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). 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). PGE_2 was separated by solid phase extraction on reversed phase (RP)-C18 material using acetonitrile (200 μl) as eluent and analyzed by RP-HPLC (30% acetonitrile/70% water + 0.007% TFA (v/v)) with UV detection at 195 nm. 11β-PGE_2 was used as internal standard to quantify PGE_2 product formation by integration of the area under the peaks.

2.8. Determination of PGE_2, 6-keto $PGF_{1\alpha}$, and 12-HHT 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 venipuncture and collected in syringes containing heparin (20 U/ml). For determination of PGE_2 and 6-keto $PGF_{1\alpha}$, aliquots of whole blood (0.8 ml) were mixed with the thromboxane synthase inhibitor CV4151 (1 μM) and with aspirin (50 μM). A total volume of 1 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 lipopolysaccharide (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 $PGF_{1\alpha}$ was quantified in the supernatant using a 6-keto $PGF_{1\alpha}$ High Sensitivity EIA Kit (Assay Designs, Ann Arbor, MI) according to the manufacturer's protocol. PGE_2 was determined as described [25]. 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 HPLC, analysis of PGE_2 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.

For determination of 12-HHT, human whole blood (2 ml) was pre-incubated with the indicated compounds at 37 °C for 5 min, and formation of 12-HHT was initiated by addition of 30 μM Ca^{2+}-ionophore A23187 and 100 μM AA. After 10 min at 37 °C, the reaction was stopped on ice, and the samples were centrifuged (600 × *g*/10 min/4 °C). Aliquots of the resulting plasma (500 μl) were then mixed with 2 ml of methanol, and 200 ng of PGB_1 was added as internal standard. The samples were placed at −20 °C for 2 h and centrifuged again (600 × g/15 min/4 °C). The supernatants were collected and diluted with 2.5 ml PBS and 75 μl HCl 1N. Formed 12-HHT was extracted and analyzed by HPLC as described [23].

2.9. Expression of human recombinant 5-lipoxygenase in Escherichia coli, preparation of homogenates and 100,000 × g supernatants, and semi-purification of 5-lipoxygenase protein

E. coli MV1190 was transformed with pT3-5-LO plasmid, and recombinant 5-LO protein was expressed at 27 °C as described [27]. Cells were lysed in 50 mM triethanolamine/HCl pH 8.0, 5 mM EDTA, soybean trypsin inhibitor (60 μg/ml), 1 mM phenylmethanesulphonyl fluoride, and lysozyme (500 μg/ml), homogenized by sonication (3 × 15 s), and centrifuged at 100,000 × *g* for 70 min at 4 °C. The 100,000 × *g* supernatant (S100) was applied to an ATP-agarose column to partially purify 5-LO as described previously [28]. S100 or semi-purified 5-LO was immediately used for activity assays.

2.10. Determination of 5-lipoxygenase product formation in cell-free systems

Aliquots of S100 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 [24]. 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.

2.11. Determination of 5-lipoxygenase product formation in intact cells

Freshly isolated neutrophils (5×10^6 ml^{-1} PGC buffer) were pre-incubated with the test compounds for 15 min at room temperature 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 1N 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 [24]. 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.

2.12. 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 garcinol on arachidonic acid release from human neutrophils

Previously, it was suggested that garcinol interferes with eicosanoid formation at the level of gene expression (COX-2) [8], or ERK1/2 phosphorylation resulting in reduced AA release in lipopolysaccharide-stimulated RAW264.7 murine macrophages [10]. We investigated the effects of garcinol on cellular $cPLA_2$-mediated AA release in human neutrophils using the Ca^{2+}-ionophore A23187 as stimulus that acts by substantial elevation of $[Ca^{2+}]_i$, independent of cellular signalling pathways. Neutrophils, pre-incubated with garcinol (3–33 μM), were stimulated with Ca^{2+}-ionophore (2.5 μM), and the released AA was derivatized with 2,4-dimethoxyaniline and analyzed by RP-HPLC. Garcinol failed to significantly suppress the release of AA up to 33 μM (Fig. 1B), whereas the $cPLA_2\alpha$-inhibitor (control) potently inhibited AA liberation.

3.2. Effects of garcinol on cellular prostanoid formation

Next, we investigated the effects of garcinol on the transformation of AA to prostanoids in intact cells. Prostanoid formation was initiated by addition of exogenous AA in order to circumvent endogenous AA supply, and thus, to exclude inhibition of prostanoid formation at the level of PLA_2-mediated substrate release. For determination of COX-1 inhibition, human platelets were pre-incubated with garcinol (1–33 μM), and formation of COX-1-derived TxB_2 and 12-HHT (non-enzymatically formed from PGH_2 in platelets [29]) was initiated by AA (5 μM). Garcinol significantly suppressed TxB_2 (Fig. 1C) and 12-HHT (Fig. 1D) formation at 10 μM with an IC_{50} of 16 and 11 μM, respectively. To investigate inhibition of cellular COX-2 activity, we assessed the formation of PGE_2 and the stable PGI_2 degradation product 6-keto $PGF_{1\alpha}$ (as biomarker for PGI_2 formation) in A549 cells. For measuring 6-keto $PGF_{1\alpha}$, higher concentrations of AA (30 instead of 1 μM) were required than for determination of PGE_2 to get detectable amounts of 6-keto $PGF_{1\alpha}$ (detection limit: 14 ng/ml at a signal to noise ratio of 3). A contribution of COX-1 can be excluded in interleukin-1β-stimulated A549 cells because COX-1 is neither detectable on the protein nor on the RNA level [30,31]. COX-2-mediated synthesis of PGE_2 was significantly inhibited at 10 μM with an apparent IC_{50} of approx. 10 μM (Fig. 2A), whereas the synthesis of 6-keto $PGF_{1\alpha}$ was not significantly reduced by garcinol

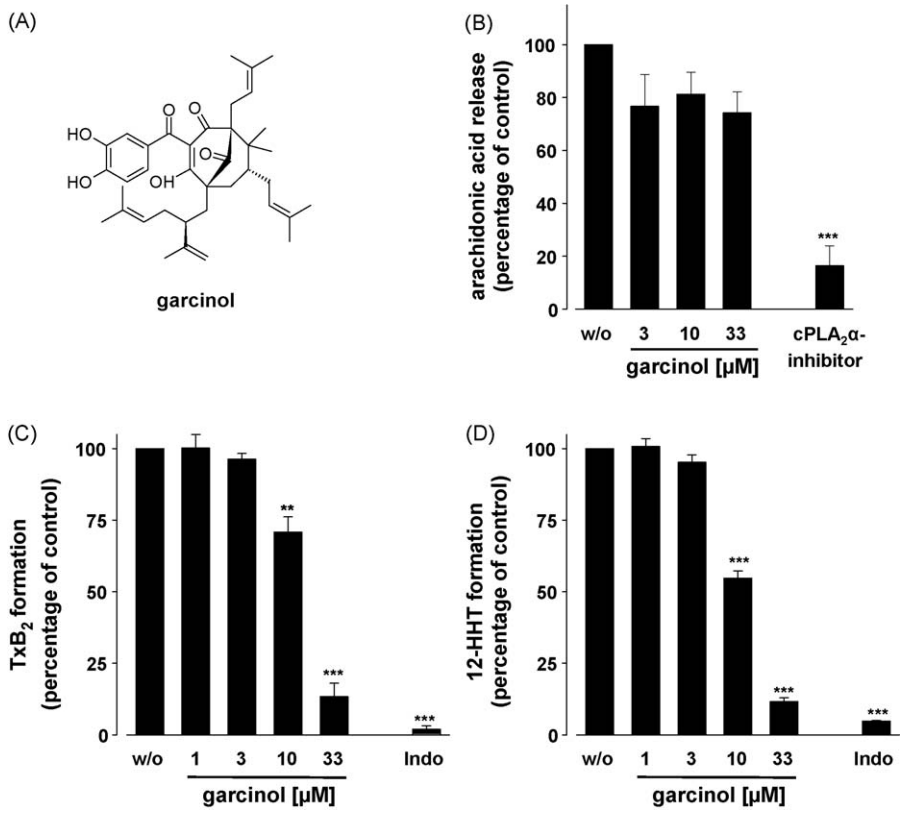

Fig. 1. Effects of garcinol on cellular arachidonic acid release and prostanoid formation. (A) Chemical structure of garcinol. (B) AA release from neutrophils. Neutrophils (3×10^7 ml^{-1} PGC buffer) were pre-incubated with the indicated concentrations of garcinol or vehicle (DMSO, w/o) for 10 min. Then, AA release was initiated by Ca^{2+}-ionophore (2.5 μM). After 5 min at 37 °C, AA was coupled to 2,4-dimethoxyaniline and analyzed by HPLC as described in Section 2. In the absence of test compounds (100%, control), 180 ng/ml AA were released. $cPLA_2\alpha$-inhibitor (10 μM) was used as control. (C) TxB_2 and (D) 12-HHT formation in intact platelets. Platelets (10^8 ml^{-1} PBS containing 1 mM $CaCl_2$) were pre-incubated with the indicated concentrations of garcinol or vehicle (DMSO, w/o) for 5 min prior to stimulation with AA (5 μM). After another 5 min at 37 °C, the formation of TxB_2 was assessed by ELISA. TxB_2 formed in the absence of test compounds (100%, control) was 360 ng/ml. The formation of 12-HHT was determined by RP-HPLC as described. 12-HHT formed in the absence of test compounds (100%, control) was 290 ng/ml. Indomethacin (Indo, 10 μM) was used as control. 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.

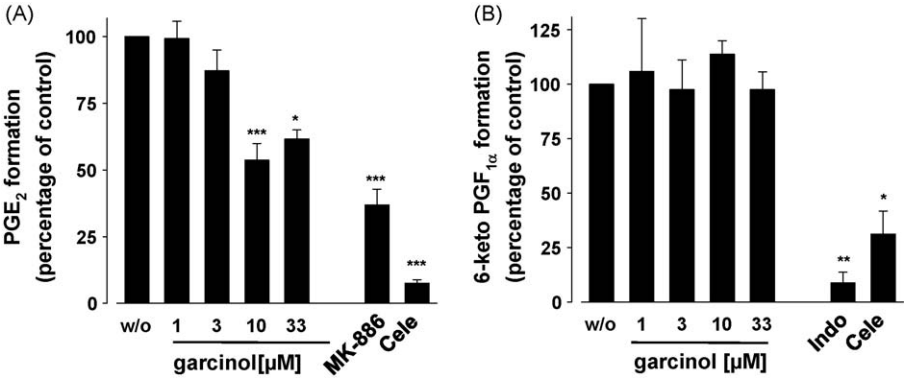

Fig. 2. Effects of garcinol on cellular arachidonic acid release and prostanoid formation. (A) PGE_2 formation in A549 cells. Interleukin-1β-stimulated A549 cells (4×10^6 ml^{-1}) were pre-incubated with garcinol or vehicle (DMSO, w/o) for 10 min, and then, 2.5 μM A23187 plus 1 μM AA and [3H]AA (18.4 kBq) were added. After 15 min at 37 °C, formed [^{3}H]PGE_2 was analyzed by RP-HPLC and liquid scintillation counting as described. The 100% value corresponds to 30 ng/ml PGE_2. MK-886 (33 μM) and celecoxib (Cele, 5 μM) were used as controls. (B) 6-Keto $PGF_{1\alpha}$ formation in A549 cells. Interleukin-1β-stimulated A549 cells (1×10^6 ml^{-1}) were pre-incubated with the indicated concentrations of garcinol or vehicle (DMSO) for 15 min prior to addition of 30 μM AA. After 15 min at 37 °C the amount of 6-keto $PGF_{1\alpha}$ was assessed by ELISA as described. The 100% value corresponds to 70 ng/ml 6-keto $PGF_{1\alpha}$. Indomethacin (Indo, 10 μM) and celecoxib (Cele, 5 μM) were used as control. Data are given as mean ± S.E., $n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$ or $^{***}p < 0.001$ vs. vehicle (0.1% DMSO) control, ANOVA + Tukey HSD *post hoc* tests.

up to 33 μM (Fig. 2B). PGE_2 formation is inhibited by garcinol within a narrow concentration range (3–10 μM), and higher concentrations of garcinol failed to completely block PGE_2 synthesis. Also MK-886 (33 μM), a mPGES-1 inhibitor, did not interfere with 6-keto $PGF_{1\alpha}$ synthesis and failed to entirely suppress PGE_2 formation [25]. In contrast, the COX-2 inhibitor celecoxib (5 μM) efficiently suppressed both formation of PGE_2 (Fig. 2A) and 6-keto $PGF_{1\alpha}$ (Fig. 2B), respectively.

3.3. Effects of garcinol on the activity of COX-1/2 in cell-free assays

We determined whether garcinol may directly interfere with the catalytic activity of isolated COX-1 and/or COX-2. The formation of 12-HHT, the major COX-1/2-derived product under the experimental conditions chosen [32], was initiated by addition of AA (5 μM for COX-1, 2 μM for COX-2). Garcinol concentration-dependently and significantly inhibited COX-1-mediated 12-HHT formation at 10 μM with an IC_{50} = 12 μM, which is in good agreement with the efficacy in intact platelets (IC_{50} = 11 μM, see above). In contrast, COX-2 activity was not significantly reduced up to 33 μM (Fig. 3A). Indomethacin (10 μM, for COX-1) and celecoxib (5 μM, for COX-2), used as reference compounds, potently inhibited 12-HHT formation, as expected (Fig. 3B).

3.4. Effects of garcinol on the activity of mPGES-1

Because garcinol failed to inhibit COX-2 in cell-free assays and did not suppress 6-keto $PGF_{1\alpha}$ synthesis in A549 cells but reduced PGE_2 formation, it appeared reasonable that garcinol might selectively suppress PGE_2 formation via inhibition of PGE_2 synthases downstream of COX-2. Microsomal preparations of interleukin-1β-stimulated A549 cells, used as source of mPGES-1 [33], were pre-incubated with garcinol or vehicle (DMSO) for 15 min, and then, PGE_2 formation was initiated by addition of 20 μM PGH_2. In agreement with the literature [25,34], the mPGES-1 inhibitor MK-886 (used as control) inhibited PGE_2 formation with an IC_{50} of 2.1 μM (data not shown). Garcinol was slightly superior over MK-886 and concentration-dependently inhibited PGE_2 formation with an IC_{50} of 1.2 μM (Fig. 4A). Decreasing the PGH_2 concentration to 1 μM even enhanced the potency of garcinol to suppress mPGES-1 activity (IC_{50} = 0.3 μM, Fig. 4B) suggesting a substrate competitive inhibitory mechanism.

To investigate whether garcinol inhibits PGE_2 synthesis in a reversible manner, microsomal preparations of A549 cells were pre-incubated with garcinol and MK-886, and wash-out experiments were carried out. MK-886 and garcinol failed to efficiently block PGE_2 synthesis at 0.3 μM, whereas PGE_2 formation was

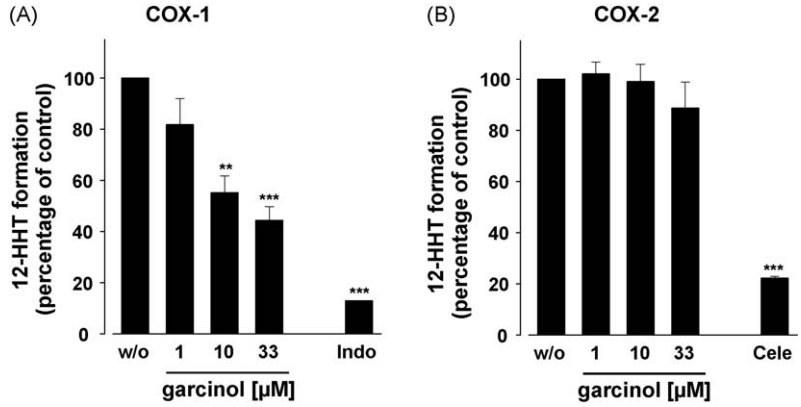

Fig. 3. Effects of garcinol on the activity of isolated COX-1 and -2. (A) Purified ovine COX-1 (50 units) or (B) human recombinant COX-2 (20 units) were added to a COX reaction mix, containing 5 mM glutathione. After pre-incubation with the test compounds or vehicle (DMSO, w/o) for 5 min, the reaction was started with 5 μM (COX-1) or 2 μM (COX-2) AA. After 5 min at 37 °C, the formation of 12-HHT was determined by RP-HPLC as described. The 100% values correspond to 80 ng/ml (COX-1) or 70 ng/ml (COX-2) 12-HHT. Indomethacin (Indo, 10 μM) and celecoxib (Cele, 5 μM) were used as controls. Data are given as mean ± S.E., $n = 3$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. vehicle (0.1% DMSO) control, ANOVA + Tukey HSD post hoc tests.

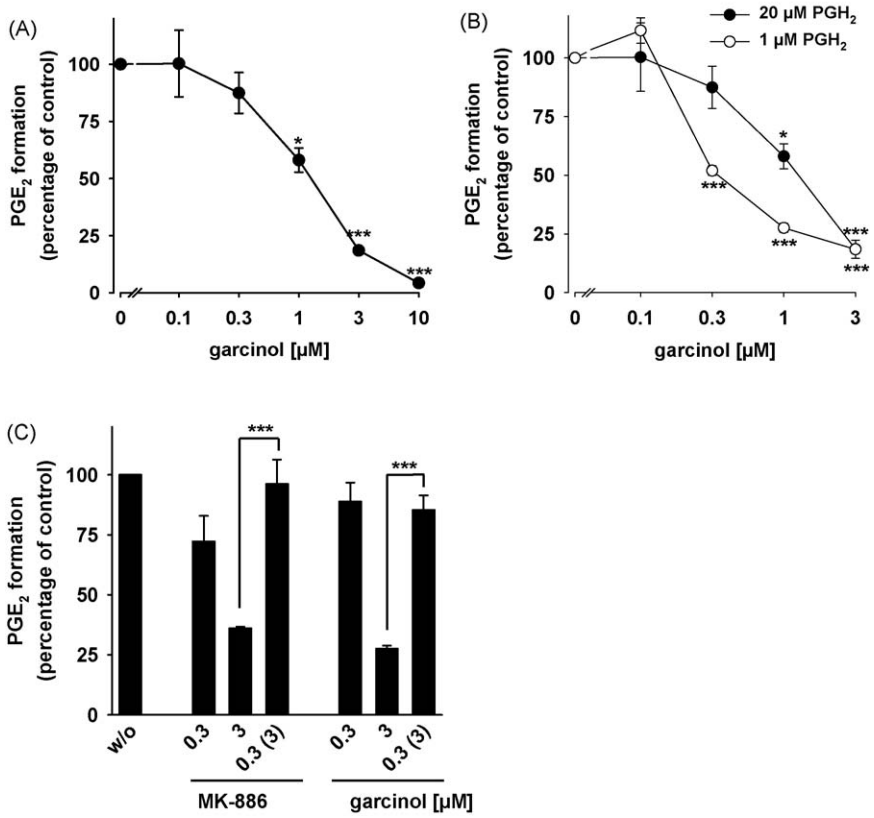

Fig. 4. Effects of garcinol on the activity of mPGES-1. (A) Concentration–response curves for garcinol. Microsomal preparations of interleukin-1β-stimulated A549 cells were pre-incubated with vehicle (DMSO) or the test compounds at the indicated concentrations for 15 min at 4 °C, and the reaction was 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 0.8 nmol PGE_2. (B) The potency of garcinol for mPGES-1 inhibition was compared at 1 and 20 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. The 100% value corresponds to 34 pmol PGE_2. (C) Reversibility of mPGES-1 inhibition by garcinol and MK-886. Microsomal preparations of interleukin-1β-stimulated A549 cells were pre-incubated with 3 μM inhibitor for 15 min at 4 °C. An aliquot was diluted 10-fold to obtain an inhibitor concentration of 0.3 μM. For comparison, microsomal preparations were pre-incubated for 15 min with vehicle (DMSO, w/o) or 0.3 μM MK-886 or garcinol, 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 RP-HPLC. Data are given as mean + S.E., $n = 2$–4, $^{*}p < 0.05$ or $^{***}p < 0.001$ vs. vehicle (0.1% DMSO) control, ANOVA + Tukey HSD *post hoc* tests.

efficiently inhibited at 3 μM (Fig. 4C). However, 10-fold dilution of the samples containing 3 μM garcinol and MK-886 restored mPGES-1 activity (Fig. 4C) implying a reversible mode of inhibition.

3.5. Effects of garcinol on prostanoid formation in human whole blood

Many potent inhibitors of prostanoid biosynthesis in cell-free assays or in intact isolated cells lose their efficacy under physiologically relevant conditions such as in whole blood. Therefore, we investigated whether garcinol could affect prostanoid biosynthesis also in a human whole blood assay. To assess inhibition of COX-1, freshly drawn human venous blood was pre-incubated with the test compounds or vehicle (DMSO) for 5 min and then treated with Ca^{2+}-ionophore plus AA (100 μM). Formation of 12-HHT, which mainly derives from constitutively expressed COX-1 under these experimental conditions, was not significantly affected by garcinol up to 33 μM (Fig. 5A). In control experiments, indomethacin (20 μM) almost completely blocked 12-HHT synthesis.

For determination of COX-2 inhibition, heparinized blood was pre-incubated with garcinol or vehicle (5 min) and stimulated with lipopolysaccharide for 5 h. Then, the levels of PGE_2 and 6-keto $PGF_{1\alpha}$ were determined in the corresponding plasma by ELISA. For measuring PGE_2 levels, PGE_2 was first separated from other AA metabolites by RP-HPLC [25]. In agreement with the results obtained with A549 cells, garcinol suppressed PGE_2 formation in whole blood in a concentration-dependent manner starting at 3 μM with an apparent IC_{50} value of 30 μM (Fig. 5B). Significant inhibition of PGE_2 formation was only reached at 30 μM for both garcinol and the mPGES-1 reference inhibitor MK-886, and also prolonged stimulation of whole blood with LPS up to 24 h did not increase the potency of garcinol. Neither garcinol nor MK-886 reduced 6-keto $PGF_{1\alpha}$ formation up to 30 μM under the same conditions, suggesting a selective interference of garcinol with PGE_2 synthesis (Fig. 5C). Note that garcinol as well as MK-886 were less efficient in inhibiting PGE_2 formation as compared to indomethacin or celecoxib.

3.6. Inhibition of 5-lipoxygenase product formation by garcinol

To determine the effects of garcinol on 5-LO product formation in intact cells, freshly isolated human neutrophils were pre-incubated with garcinol for 15 min and 5-LO product synthesis was initiated by Ca^{2+}-ionophore A23187 (2.5 μM) plus exogenous AA (20 μM). 5-LO product formation was concentration-dependently inhibited by garcinol with an $IC_{50} = 1.9$ μM (Fig. 6A). Differences in the potency of garcinol to inhibit the formation of LTB_4 (formed via LTA_4 hydrolase) and 5(*S*)-hydro(pero)xy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid ($IC_{50} = 1.5$ and 1.8 μM, respectively) were not observed, excluding an interference of garcinol with LTA_4 hydrolase at these concentrations. The efficiency of garcinol was somewhat improved in a cell-free assay using 100,000 × *g* supernatant of homogenized neutrophils as source of enzyme

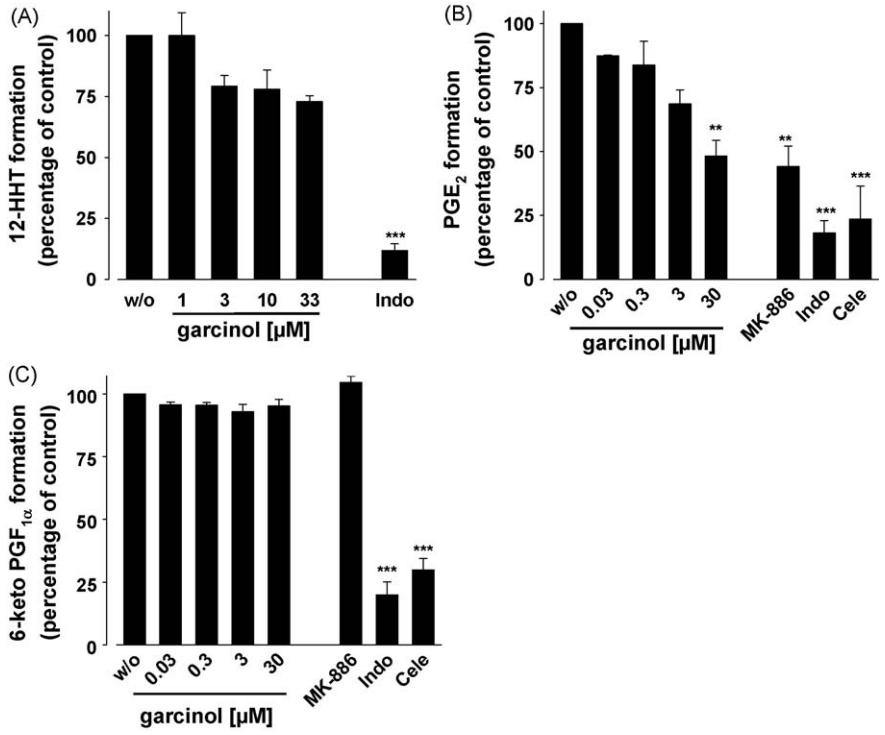

Fig. 5. Effects of garcinol on prostanoid formation in human whole blood. (A) 12-HHT formation. Heparinized whole blood was pre-incubated with garcinol or vehicle (DMSO, w/o) at the indicated concentrations for 5 min, and AA (100 μM) and Ca^{2+}-ionophore (30 μM) were added to induce COX product formation. After 10 min at 37 °C, 12-HHT was extracted and analyzed by RP-HPLC as described. The 100% value corresponds to 1.0 μg/ml 12-HHT. Indomethacin (Indo, 20 μM) was used as control. (B and C) Heparinized human whole blood, treated with 1 μM thromboxane synthase inhibitor and 50 μM aspirin, was pre-incubated with the indicated concentrations of garcinol or vehicle (DMSO, w/o) for 5 min at room temperature, and then, prostanoid formation was induced by addition of 10 μg/ml lipo polysaccharide. After 5 h at 37 °C, (B) PGE_2 was extracted from plasma by RP-18 solid phase extraction, separated by RP-HPLC, and quantified by ELISA as described. The 100% value corresponds to 9 ng/ml PGE_2. (C) 6-keto $PGF_{1\alpha}$ was directly determined in blood plasma by ELISA. The 100% value corresponds to 4 ng/ml 6-keto $PGF_{1\alpha}$. MK-886 (30 μM), indomethacin (Indo, 50 μM), celecoxib (Cele, 20 μM), or vehicle (DMSO) was used as controls. 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.

(IC_{50} = 0.5 μM, Fig. 6B) and was even higher for semi-purified recombinant 5-LO (IC_{50} = 0.1 μM, Fig. 6C). In contrast, the iron-ligand type 5-LO inhibitor BWA4C (1 μM, used as control) was equally effective independent of the assay conditions (data not shown).

4. Discussion

Animal studies have revealed an anti-carcinogenic potential of garcinol [2,3], and *in vitro* studies have demonstrated potent growth-inhibitory and apoptotic [5,6] as well as anti-oxidant [4] and anti-inflammatory effects [8,10]. Interference with lipopolysaccharide-induced NFκB signalling, lowering COX-2 and iNOS expression, may explain the long-term anti-inflammatory and anti-carcinogenic effects of garcinol [8], whereas the short-term effects were ascribed to impaired $cPLA_2$ activation through inhibition of ERK-1/2 phosphorylation restricting the supply of AA for eicosanoid formation [10]. Although suppression of eicosanoid formation may be a crucial mode of action of garcinol, direct inhibitory effects on enzymes involved in eicosanoid biosynthesis have not been investigated yet.

Our data demonstrate that garcinol is a direct and potent inhibitor of purified 5-LO and cell-free mPGES-1 with IC_{50} = 0.1 and 0.3 μM, respectively, and also a direct inhibition of COX-1 by garcinol is evident. Inhibition of other molecular targets (i.e., histone acetyltransferase p300 [11], P300/CBP-associated factor [11], and acetylcholine esterase [12]) required higher concentrations of garcinol (IC_{50} = 0.7–7 μM). However, because of the different experimental settings applied for analysis of the interference of garcinol with these targets, direct comparison of garcinol's potencies is difficult. 5-LO and mPGES-1 are crucial enzymes in the biosynthesis of key mediators in inflammatory processes [16,19], providing a rationale for the anti-inflammatory effects of garcinol. Moreover, 5-LO and mPGES-1 are overexpressed in many tumors, and PGE_2 as well as 5-LO products play important roles in tumorigenesis [16,18,19]. Together, we conclude that the interference of garcinol with 5-LO and mPGES-1 provides a molecular basis for its documented anti-carcinogenic and anti-inflammatory properties.

Inhibition of 5-LO and mPGES-1 is evident also in intact cell assays and in human whole blood. On the other hand, inhibition of COX-1-derived 12-HHT formation in human whole blood was minute and not significant up to 33 μM, although COX-1 activity is obviously inhibited in intact platelets and in the cell-free assay (IC_{50} = 11 and 12 μM, respectively). This loss of potency in whole blood might be related to albumin-binding of garcinol but also to competition with high AA concentrations (that are required in whole blood for analysis of 12-HHT formation) for binding to the active site of COX-1.

The experimental settings (stimuli used, availability of respective substrates, and products determined) of these cell-based assays allowed us to attribute the effects of garcinol to the interference with the putative targets and to exclude other points of attack. In particular, garcinol may simply block the supply of AA by interference with $cPLA_2$ activity, an enzyme, whose activation and cellular functionality has been previously proposed to be affected by garcinol [10]. Hong et al. showed that in lipopolysaccharide-stimulated RAW264.7 murine macrophages garcinol blocked AA release by impairing the activation of $cPLA_2$. This was ascribed to the interference with defined signalling steps (i.e., ERK

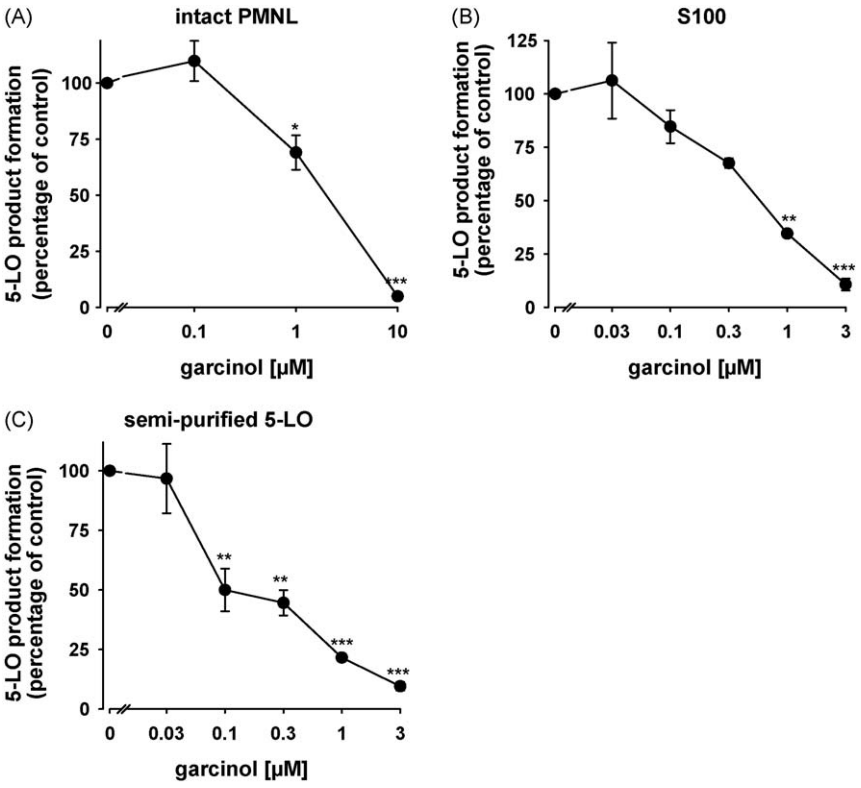

Fig. 6. Inhibition of 5-lipoxygenase activity by garcinol. (A) Neutrophils (5×10^6 cells/ml) were pre-incubated with the indicated concentrations of garcinol or vehicle (DMSO) for 15 min. 5-LO product formation was initiated by addition of 2.5 μM Ca^{2+}-ionophore plus 20 μM AA. (B) S100 of *E. coli* lysates expressing human recombinant 5-LO or (C) semi-purified human recombinant 5-LO were supplemented with garcinol and 1 mM ATP, and product formation was started by addition of 2 mM $CaCl_2$ and 20 μM AA. The 100% values correspond to 1.8, 1.3, and 1.4 μg/ml 5-LO products for intact cells, S100, and purified enzyme, respectively. Data are given as mean + S.E., $n = 3–6$, $^{*}p < 0.05$, $^{**}p < 0.01$ or $^{***}p < 0.001$ vs. vehicle (0.1% DMSO) control, ANOVA + Tukey HSD *post hoc* tests.

signalling, phosphorylation of $cPLA_2$) [10]. To circumvent such signalling pathways for $cPLA_2$ activation, we challenged human neutrophils with the Ca^{2+}-ionophore A23187, where the massive increase in intracellular Ca^{2+} essentially stimulates $cPLA_2$. Garcinol failed to block AA liberation under these experimental conditions, indicating that PLA_2-mediated AA release is not generally inhibited by garcinol. Note that in our cell-based assays analyzing AA transformations by COX-1/2 or by 5-LO, AA was exogenously supplemented to avoid effects on eicosanoid formation due to inhibition of PLA_2. On the other hand, the effects of garcinol on PGE_2 and 6-keto $PGF_{1\alpha}$ formation in whole blood were analyzed under conditions (lipopolysaccharide challenge) where AA was supplied by endogenous sources. In contrast to PGE_2 formation, the synthesis of 6-keto $PGF_{1\alpha}$ was unaffected by garcinol, excluding general suppression of AA and suggesting PGE_2 synthase (i.e., mPGES-1) as molecular target of garcinol. However, our data cannot exclude that garcinol also inhibits other PGE_2 synthases (such as cytosolic PGE_2 synthase or mPGES-2) in addition to mPGES-1.

PGE_2 and 6-keto $PGF_{1\alpha}$ are synthesized via the COX-2 pathway in lipopolysaccharide-stimulated whole blood [35]. Because 6-keto $PGF_{1\alpha}$ formation in whole blood was unaffected by garcinol, our data imply that garcinol-mediated suppression of PGE_2 synthesis in the same assay is independent of an interference with COX-2. Also, garcinol failed to inhibit isolated COX-2. Co-expression studies have revealed a preferred functional coupling of COX-2 and mPGES-1 [36] suggesting that mPGES-1 is the major contributing PGE_2 synthase for PGE_2 synthesis in lipopolysaccharide-stimulated blood. Nevertheless, in analogy to other mPGES-1 inhibitors [25], garcinol failed to completely suppress PGE_2 synthesis in whole blood and the remaining PGE_2 synthesis of approx. 40% might be attributable to other PGE_2 synthases (i.e., cytosolic PGE_2 synthase or mPGES-2) [25].

For inhibition of purified 5-LO (IC_{50} = 0.1 μM) and cellular 5-LO product formation (IC_{50} = 1.9 μM), a discrepancy in the potencies of garcinol was obvious. Interestingly, a similar pattern was found for the structurally related hyperforin [23] that possesses a unique inhibitory mode of action by interfering with the membrane-binding region of 5-LO's C2-like domain (Feißt et al., 2009, manuscript submitted). However, garcinol is an effective anti-oxidant [4] and may act as redox-type inhibitor of 5-LO reducing the active site iron. Such reduction might be more vigorous for cell-free 5-LO as compared to intracellular enzyme, a common phenomenon observed for many redox-active 5-LO inhibitors [37].

Despite the obvious inhibition of PGE_2 and LT formation by garcinol *in vitro*, the question whether the interference of garcinol with mPGES-1 and 5-LO accounts for its anti-inflammatory and anti-tumorigenic properties *in vivo*, cannot be clearly answered yet. Unpublished studies, mentioned by Hong et al. [10], showed that garcinol inhibited phorbol myristate acetate-induced mouse ear edema accompanied by decreased PGE_2 and LTB_4 production. Of interest, in these experiments the peak plasma levels of garcinol (12 μM) obtained after oral application of garcinol were in a concentration range, where cellular inhibition 5-LO and mPGES-1 is evident in our study. Therefore, one may speculate that garcinol, after oral administration, could achieve plasma levels sufficient to markedly suppress PGE_2 and LT biosynthesis under inflammatory conditions. PGE_2 is one of the most established key players in the initiation and progression of inflammation [38], and NSAIDs exert their beneficial effects *in vivo* essentially by repressing PGE_2 formation [13]. Animals deficient in COX-2 or mPGES-1 clearly show reduced inflammatory symptoms [19,39]. Moreover, LTB_4 is

a potent chemotactic and chemokinetic agent that recruits pro-inflammatory cells towards sites of inflammation [40]. But also tumorigenesis, particularly of colon, breast, prostate, and lung carcinoma has been associated with excessive PGE_2 and LT formation [16,18,19]. COX-2 selective inhibitors have shown a chemopreventive potential [41], and mPGES-1 is overexpressed in various cancers such as non-small cell lung cancer, invasive breast cancer, colorectal cancer, and gastric cancer [19]. Also 5-LO and its helper-protein FLAP are overexpressed in cancer cells, and 5-LO products promote cell proliferation and/or inhibit apoptosis, whereas 5-LO or FLAP inhibitors caused cell death [18].

In conclusion, we have identified human mPGES-1 and 5-LO as molecular targets of garcinol, and we showed that garcinol potently interferes with the catalytic activity of these enzymes in cell-free and cell-based assays. Inhibition of mPGES-1 and 5-LO may contribute to the anti-carcinogenic and anti-inflammatory effectiveness of garcinol observed in previous studies. Our data indicate that garcinol exerts a promising pharmacological profile combining potent inhibition of PGE_2 and LT formation without marked inhibition of COX isoenzymes.

Conflict of interest statement

None.

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