

RESEARCH PAPER

Myrtucommulone, a natural acylphloroglucinol, inhibits microsomal prostaglandin E₂ synthase-1

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Background and purpose: The selective inhibition of prostaglandin (PG)E₂ formation via interference with microsomal PGE₂ synthase (mPGES)-1 could have advantages in the treatment of PGE₂-associated diseases, such as inflammation, fever and pain, compared with a general suppression of all PG biosynthesis, provided by inhibition of cyclooxygenase (COX)-1 and 2. Here, we addressed whether the naturally occurring acylphloroglucinol myrtucommulone (MC) from *Myrtus communis* L. (myrtle) affected mPGES-1.

Experimental approach: The effect of MC on PGE₂ formation was investigated in a cell-free assay by using microsomal preparations of interleukin-1 β -stimulated A549 cells as the source of mPGES-1, in intact A549 cells, and in lipopolysaccharide-stimulated human whole blood. Inhibition of COX-1 and COX-2 activity in cellular and cell-free assays was assessed by measuring 12(S)-hydroxy-5-cis-8,10-trans-heptadecatrienoic acid and 6-oxo PGF_{1 α} formation.

Key results: MC concentration-dependently inhibited cell-free mPGES-1-mediated conversion of PGH₂ to PGE₂ (IC₅₀ = 1 μ mol·L⁻¹). PGE₂ formation was also diminished in intact A549 cells as well as in human whole blood at low micromolar concentrations. Neither COX-2 activity in A549 cells nor isolated human recombinant COX-2 was significantly affected by MC up to 30 μ mol·L⁻¹, and only moderate inhibition of cellular or cell-free COX-1 was evident (IC₅₀ > 15 μ mol·L⁻¹).

Conclusions and implications: MC is the first natural product to inhibit mPGES-1 that efficiently suppresses PGE₂ formation without significant inhibition of the COX enzymes. This provides an interesting pharmacological profile suitable for interventions in inflammatory disorders, without the typical side effects of coxibs and non-steroidal anti-inflammatory drugs.

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Abbreviations: 12-HHT, 12(S)-hydroxy-5-cis-8,10-trans-heptadecatrienoic acid; COX, cyclooxygenase; cPGES, cytosolic prostaglandin E₂ synthase; cPLA₂, cytosolic phospholipase A₂; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; MAPEG, membrane-associated proteins involved in eicosanoid and glutathione metabolism; MC, myrtucommulone; mPGES, microsomal prostaglandin E₂ synthase; NSAIDs, non-steroidal anti-inflammatory drugs; PG, prostaglandin

Introduction

Prostaglandin (PG)E₂ is a prominent product of the cyclooxygenase (COX) pathway and has long been considered as a mediator of inflammation, pain, fever and cancer, but it is also known to regulate physiological functions in the gastrointestinal tract, the kidney and in the immune and nervous systems (Smith, 1989; Sugimoto and Narumiya,

2007). Three terminal isoforms of PGE₂ synthases (PGES) have been identified so far, and co-transfection of COX-1 and 2 with PGES isoenzymes in mammalian cells suggests that molecular interactions may cause preferential functional coupling (Murakami *et al.*, 2002; Samuelsson *et al.*, 2007). Thus, the constitutively expressed cytosolic PGE₂ synthase (cPGES) was found to be coupled to COX-1 being necessary for homeostasis, whereas the microsomal PGE₂ synthase (mPGES)-1 is the predominant isoform involved in COX-2-mediated PGE₂ production, responsible for distinct pathophysiological effects (Park *et al.*, 2006). mPGES-1 belongs to the membrane-associated proteins involved in eicosanoid and glutathione metabolism (MAPEG) family, and its expression

is induced by pro-inflammatory stimuli (such as interleukin-1 β) congruent with expression of COX-2 (Jakobsson *et al.*, 1999). Knockout (KO) experiments have revealed that mice deficient in mPGES-1 develop normally but exhibit reduced inflammatory responses (Trebino *et al.*, 2003) and do not show fever in response to lipopolysaccharide (Engblom *et al.*, 2003).

Selective inhibition of inflammatory PGE₂ formation is considered superior over inhibition of all COX-derived products (Samuelsson *et al.*, 2007), as other prostanoids such as PGI₂ and PGD₂ possess important physiological functions (Funk, 2001). Thus, mPGES-1 may constitute a potential target for therapeutic intervention.

Only a few inhibitors of mPGES-1 have been described, including polyunsaturated fatty acids, such as arachidonic acid and 15-deoxy- $\Delta^{12,14}$ -PGJ₂ (IC₅₀ = 0.3 $\mu\text{mol}\cdot\text{L}^{-1}$, (Quraishi *et al.*, 2002)). Further, mPGES-1 is inhibited by MK-886 [3-[1-(4-chlorobenzyl)-3-t-butyl-thio-5-isopropylindol-2-yl]-2,2-dimethylpropanoic acid, IC₅₀ = 2.4 $\mu\text{mol}\cdot\text{L}^{-1}$, (Claveau *et al.*, 2003)] and its structural derivatives (Riendeau *et al.*, 2005)

including licoferone (Koeberle *et al.*, 2008). Synthetic phenanthrene imidazoles constitute another group of potent, selective, and orally active mPGES-1 inhibitors with IC₅₀ values of 0.42 and 1.3 $\mu\text{mol}\cdot\text{L}^{-1}$ in A549 cells and human whole blood respectively (Cote *et al.*, 2007; Xu *et al.*, 2008).

Myrtucommulone (MC, Figure 1A) consists of an isobutyrophenone core (IBP-C), substituted with two syncarpic acid moieties and represents an oligomeric, nonprenylated acylphloroglucinol which is present in the leaves of myrtle (*Myrtus communis* L., Myrtaceae) (Appendino *et al.*, 2002). Myrtle is used in traditional folk medicine as an antiseptic agent, and extracts of myrtle have been reported to possess antibacterial (Bonjar, 2004), analgesic (Levesque and Lafont, 2000) and antioxidative properties (Rosa *et al.*, 2003; Romani *et al.*, 2004). MC acts as antioxidant (Rosa *et al.*, 2003), shows antibacterial activity against clinically relevant bacteria (Appendino *et al.*, 2002), induces selective apoptosis of cancer cells (Tretiakova *et al.*, 2007) and suppresses inflammatory neutrophil functions (Feisst *et al.*, 2005). Nevertheless, little is known about the molecular targets of MC. Recently, we have

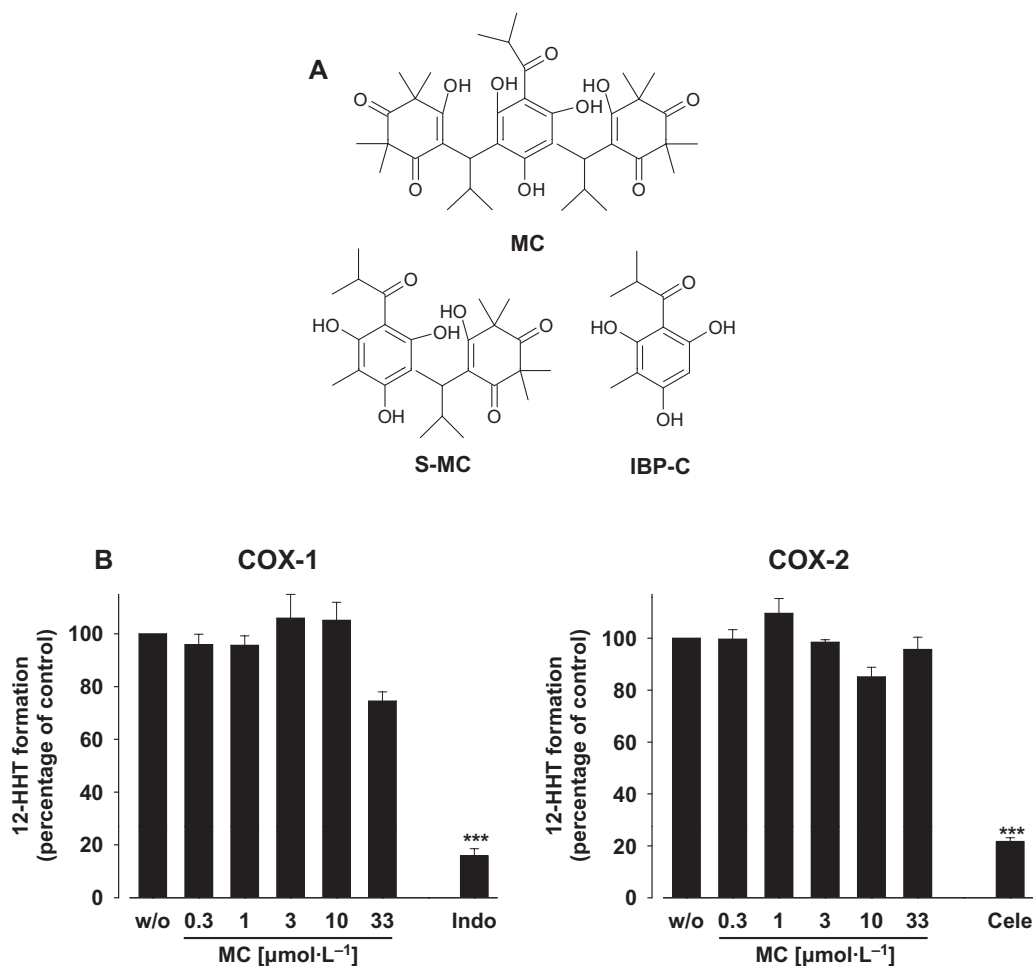


Figure 1 Effects of myrtucommulone (MC) on the activity of isolated cyclooxygenase (COX)-1 and COX-2. (A) Chemical structures of MC, semi-myrtucommulone (S-MC) and the isobutyrophenone core (IBP-C). (B) Purified ovine COX-1 (50 units) or human recombinant COX-2 (20 units) were added to a COX reaction mix. Vehicle (w/o), MC (0.3 to 33 $\mu\text{mol}\cdot\text{L}^{-1}$), indomethacin (Indo, 10 $\mu\text{mol}\cdot\text{L}^{-1}$) and celecoxib (Cele, 5 $\mu\text{mol}\cdot\text{L}^{-1}$) were added, and after 5 min the reaction was started by addition of arachidonic acid (5 $\mu\text{mol}\cdot\text{L}^{-1}$ for COX-1, 2 $\mu\text{mol}\cdot\text{L}^{-1}$ for COX-2). After another 5 min at 37°C, the formation of 12-HHT (12(S)-hydroxy-5-cis-8,10-trans-heptadecatrienoic acid) was determined by RP-HPLC as described in the *Methods* section. Data are given as mean \pm SE, $n = 3$, *** $P < 0.001$ vs. vehicle (0.1% dimethyl sulphoxide) control, ANOVA + Tukey HSD post hoc tests.

shown that 5-lipoxygenase in intact polymorphonuclear leukocytes (IC₅₀ = 2 µmol·L⁻¹) and to a lesser extent also COX-1 in human platelets (IC₅₀ = 17 µmol·L⁻¹) are directly targeted by MC (Feisst *et al.*, 2005). Here we provide strong evidence that MC potently interferes with mPGES-1.

Methods

Cells and cell viability assay

A549 cells were cultured in DMEM high glucose (4.5 g·L⁻¹) medium supplemented with heat-inactivated fetal calf serum (FCS) (10%, v,v), penicillin (100 U·mL⁻¹) and streptomycin (100 µg·mL⁻¹) at 37°C in a 5% CO₂ incubator. After 3 days, confluent cells were detached by using 1× trypsin-EDTA solution and reseeded at 2 × 10⁶ cells in 20 mL medium in 175 cm² flasks. Cell viability was measured by using the colorimetric thiazolyl blue tetrazolium (MTT) dye reduction assay. A549 cells (4 × 10⁴ cells per 100 µL medium) were plated into a 96-well microplate and incubated at 37°C and 5% CO₂ for 16 h. Then, MC, semi-myrtucommulone (S-MC) or the IBP-C (30 µmol·L⁻¹, each) were added, and the samples were incubated for another 5 h. MTT (20 µL, 5 mg·mL⁻¹) was added, and the incubations were continued for 4 h. The formazan product was solubilized with SDS (10%, m,v in 20 mmol·L⁻¹ HCl), and the absorbance of each sample was measured at 595 nm relative to the absorbance of vehicle (dimethyl sulphoxide, DMSO)-treated control cells by using a multi-well scanning spectrophotometer (Victor³ plate reader, PerkinElmer, Rodgau-Juegesheim, Germany). None of the substances significantly reduced cell viability (data not shown), excluding possible acute cytotoxic effects of the compounds in the cellular assays.

Activity assays of isolated COX-1 and 2

Inhibition of the activities of purified ovine COX-1 and human COX-2 was performed as described (Mitchell *et al.*, 1993; Capdevila *et al.*, 1995). Although the purified COX-1 is not of human origin, ovine COX-1 is generally used for inhibitor studies when examining the effectiveness of compounds on the activity of isolated COX-1 enzyme (Mitchell *et al.*, 1993). Briefly, purified COX-1 (ovine, 50 units) or COX-2 (human recombinant, 20 units) were diluted in 1 mL reaction mixture containing 100 mmol·L⁻¹ Tris buffer pH 8, 5 mmol·L⁻¹ glutathione, 5 µmol·L⁻¹ haemoglobin and 100 µmol·L⁻¹ 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 arachidonic acid (5 µmol·L⁻¹ for COX-1, 2 µmol·L⁻¹ for COX-2) was added to start the reaction. After 5 min at 37°C, the COX product 12-HHT (12(S)-hydroxy-5-cis-8,10-trans-heptadecatrienoic acid) was extracted and then analysed by high performance liquid chromatography (HPLC) as described (Guichardant *et al.*, 1989; Albert *et al.*, 2002).

Induction of mPGES-1 in A549 cells and isolation of microsomes

Preparation of A549 cells was performed as described (Koeberle *et al.*, 2008). In brief, cells (2 × 10⁶ cells in 20 mL medium) were plated in 175 cm² flasks and incubated for 16 h

at 37°C and 5% CO₂. Subsequently, the culture medium was replaced by fresh DMEM high glucose medium containing FCS (2%, v,v). In order to induce mPGES-1 expression, interleukin-1β (1 ng·mL⁻¹) was added, and cells were incubated for another 48 h. Thereafter, cells were detached with trypsin-EDTA, washed with PBS and frozen in liquid nitrogen. Ice-cold homogenization buffer (0.1 mol·L⁻¹ potassium phosphate buffer pH 7.4, 1 mmol·L⁻¹ PMSF, 60 µg·mL⁻¹ soybean trypsin inhibitor, 1 µg·mL⁻¹ leupeptin, 2.5 mmol·L⁻¹ glutathione and 250 mmol·L⁻¹ sucrose) was added and after 15 min, cells were resuspended and sonicated on ice (3 × 20 s). 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 (Bradford, 1976). Microsomal membrane fractions were stored at -80°C for several weeks.

Determination of PGE₂ synthase activity in microsomes of A549 cells

Prostaglandin E₂ synthase activity was determined as previously reported (Thoren and Jakobsson, 2000). Microsomal membranes were diluted in potassium phosphate buffer (0.1 mol·L⁻¹, pH 7.4) containing 2.5 mmol·L⁻¹ 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 PGH₂ (20 µmol·L⁻¹, final concentration). After 1 min at 4°C, the reaction was terminated by using stop solution (100 µL; 40 mmol·L⁻¹ FeCl₂, 80 mmol·L⁻¹ citric acid and 10 µmol·L⁻¹ of 11β-PGE₂). PGE₂ was separated by solid phase extraction on reversed phase (RP)-C18 material by using acetonitrile (200 µL) as eluent, and analysed by RP-HPLC [30% acetonitrile in water + 0.007% TFA (v,v), Nova-Pak® C18 column, 5 × 100 mm, 4 µm particle size, flow rate 1 mL·min⁻¹] with UV detection at 195 nm. 11β-PGE₂ was used as internal standard to quantify PGE₂ product formation by integration of the area under the peaks.

Determination of PGE₂ and 6-oxo PGF_{1α} formation in intact A549 cells

A549 cells (2 × 10⁶ cells) were plated in a 175 cm² flask and incubated for 16 h at 37°C and 5% CO₂. Then, the medium was changed, and the cells were stimulated with interleukin-1β (1 ng·mL⁻¹) in DMEM high glucose medium containing FCS (2%, v,v) for 48 h. After trypsination, cells were washed with PBS twice. For determination of PGE₂, 4 × 10⁶ cells·per·mL PBS containing CaCl₂ (1 mmol·L⁻¹) were pre-incubated with the indicated compounds at 37°C for 10 min, and PGE₂ formation was started by addition of ionophore A23187 (2.5 µmol·L⁻¹), arachidonic acid (1 µmol·L⁻¹) and [³H]arachidonic acid (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 mol·L⁻¹), and the internal standard 11β-PGE₂ (2 nmol) was added. Radiolabelled PGE₂ was separated by RP-18 solid phase extraction and eluted with acetonitrile (200 µL). Solid phase extraction and HPLC analysis were performed as described above. The amount of 11β-PGE₂ was

quantified by integration of the area under the eluted peaks. For quantification of radiolabelled PGE₂, 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-oxo PGF_{1α}, 5 × 10⁶ cells resuspended in 1 mL PBS containing CaCl₂ (1 mmol·L⁻¹) were pre-incubated with the indicated compounds for 15 min at 37°C, and 6-oxo PGF_{1α} formation was initiated by addition of arachidonic acid (30 μmol·L⁻¹). 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-oxo PGF_{1α} was assessed by enzyme-linked immunosorbent assay (ELISA) by using a monoclonal antibody against 6-oxo PGF_{1α} according to the protocol described by Yamamoto *et al.* (1987). For the ELISA, the monoclonal antibody (0.2 μg per 200 μL) was coated on microtiter plates via a goat anti-mouse immunoglobulin G antibody. 6-oxo PGF_{1α} (15 μg) was linked to bacterial β-galactosidase (0.5 mg), and the enzyme activity bound to the antibody was determined in an ELISA reader at 550 nm (reference wavelength: 630 nm) by using chlorophenol-red-β-D-galactopyranoside (CPRG, Roche Diagnostic GmbH) as substrate.

Determination of PGE₂ and 12-HHT in whole blood

Peripheral blood from healthy adult volunteers, who had not received any medication for at least 2 weeks under informed consent, was obtained by venepuncture and collected in syringes containing heparin (20 U·mL⁻¹). For determination of PGE₂, aliquots of whole blood (0.8 mL) were mixed with the thromboxane synthase inhibitor CV4151 (1 μmol·L⁻¹) and with aspirin (50 μmol·L⁻¹). A total volume of 1 mL was adjusted with sample buffer (10 mmol·L⁻¹ potassium phosphate buffer pH 7.4, 3 mmol·L⁻¹ KCl, 140 mmol·L⁻¹ NaCl and 6 mmol·L⁻¹ D-glucose). After pre-incubation with the indicated compounds for 5 min at room temperature, the samples were stimulated with lipopolysaccharide (10 μg·mL⁻¹) for 5 h at 37°C. PGE₂ formation was stopped on ice; the samples were centrifuged (2300× *g*, 10 min, 4°C), and citric acid (30 μL, 2 mol·L⁻¹) was added to the supernatant. After another centrifugation step (2300× *g*, 10 min, 4°C), solid phase extraction and HPLC analysis of PGE₂ was performed as described above. 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 by using a PGE₂ High Sensitivity EIA Kit (Assay Designs, Ann Arbor, MI) according to the manufacturer's protocol.

For determination of the COX product 12-HHT, whole blood (2 mL) was pre-incubated with the indicated compounds at 37°C for 10 min, and formation of 12-HHT was initiated by addition of 30 μmol·L⁻¹ Ca²⁺-ionophore A23187 and 100 μmol·L⁻¹ arachidonic acid. 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 PGB₁ was added as internal standard. The samples were held at -20°C for 2 h and centrifuged again (600× *g*, 15 min,

4°C). The supernatants were collected and diluted with 2.5 mL PBS and 75 μL HCl 1 N. Formed 12-HHT was extracted and analysed by HPLC as described (Albert *et al.*, 2002).

SDS-PAGE and Western blot

Cells (4 × 10⁶ cells·per·mL) were resuspended in 50 μL PBS buffer pH 7.2, mixed with the same volume of 2 × SDS-PAGE sample loading buffer [20 mmol·L⁻¹ Tris-HCl, pH 8, 2 mmol·L⁻¹ EDTA, 5% (m,v) SDS and 10% (v,v) β-mercaptoethanol] and boiled for 5 min at 95°C. Aliquots (20 μL) equivalent to 0.8 × 10⁶ cells were mixed with 4 μL glycerol, and 0.1% bromophenol blue (1:1, v,v) and proteins were separated by SDS-PAGE. 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 1:1000 dilution of alkaline phosphatase-conjugated immunoglobulin G for 3 h at room temperature. After washing, proteins were visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate.

Statistics

Data are expressed as mean ± SE. IC₅₀ values were calculated by non-linear regression by using SigmaPlot 9.0 (Systat Software Inc., San Jose, USA) one site binding competition. The programme Graphpad Instat (Graphpad Software Inc., San Diego, CA) was used for statistical comparisons. Statistical evaluation of the data was performed by one-way ANOVA for independent or correlated samples followed by Tukey HSD post hoc tests. Where appropriate, Student's *t*-test for paired and correlated samples was applied. A *P*-value of <0.05 (*) was considered significant.

Materials

Myrtucommulone and S-MC (Figure 1) were isolated from myrtle leaves as described previously (Appendino *et al.*, 2002). The IBP-C (Figure 1) was synthesized from S-MC (G. Appendino, unpubl. data). The compounds were dissolved in DMSO and kept in the dark at -20°C, and freezing-thawing cycles were kept to a minimum. The thromboxane synthase inhibitor CV4151 (Kato *et al.*, 1985) and celecoxib were generous gifts by Dr S Laufer (University of Tuebingen, Germany), and the anti-6-oxo PGF_{1α} antibody was provided by Dr T Dingermann (University of Frankfurt, Germany). DMEM high glucose (4.5 g·L⁻¹) medium, penicillin, streptomycin, trypsin-EDTA solution, sodium pyruvate were from PAA (Coelbe, Germany); PGH₂ from Larodan (Malmö, Sweden); 11β-PGE₂, PGB₁, MK-886, 6-oxo PGF_{1α}, human recombinant COX-2, isolated ovine COX-1 from Cayman Chemical (Ann Arbor, MI); [5, 6, 8, 9, 11, 12, 14, 15-³H]arachidonic acid ([³H]arachidonic acid) from BioTrend Chemicals GmbH (Cologne, Germany); Ultima Gold™ XR from Perkin Elmer (Boston, MA). All other chemicals were obtained from Sigma-Aldrich (Deisenhofen, Germany) unless stated otherwise.

Results

Effects of myrtucommulone on the activity of isolated COX-1 and 2

We have previously shown that MC suppresses cellular COX-1 product formation in intact platelets ($IC_{50} = 17 \mu\text{mol}\cdot\text{L}^{-1}$), whereas MC up to $30 \mu\text{mol}\cdot\text{L}^{-1}$ did not inhibit COX-2 activity in monocytic Mono Mac 6 cells (Feisst *et al.*, 2005). Here, we analysed the direct effects of MC on isolated COX enzymes. Purified ovine COX-1 and purified human recombinant COX-2 were pre-incubated with MC for 5 min, arachidonic acid ($5 \mu\text{mol}\cdot\text{L}^{-1}$ for COX-1, $2 \mu\text{mol}\cdot\text{L}^{-1}$ for COX-2) was added, and after 5 min at 37°C the formation of 12-HHT, which is the major COX-1 and 2-derived product under these experimental conditions (Capdevila *et al.*, 1995), was determined. MC up to $33 \mu\text{mol}\cdot\text{L}^{-1}$ caused no significant inhibition of COX-2 activity and hardly suppressed COX-1 activity ($IC_{50} > 33 \mu\text{mol}\cdot\text{L}^{-1}$) (Figure 1B), excluding a strong interaction of MC with these enzymes. In control experiments, the COX inhibitor indomethacin [$10 \mu\text{mol}\cdot\text{L}^{-1}$, (Mitchell *et al.*, 1993)] suppressed the activity of COX-1, and the selective COX-2 inhibitor celecoxib ($5 \mu\text{mol}\cdot\text{L}^{-1}$) inhibited the activity of COX-2, as expected (Figure 1B).

Effects of myrtucommulone on the activity of mPGES-1

Next, we investigated if MC interferes with mPGES-1 activity in a cell-free assay. A549 cells treated with $1 \text{ ng}\cdot\text{mL}^{-1}$ interleukin-1 β expressed mPGES-1 after 48 h, as analysed by SDS-PAGE and Western blotting (Figure 2A). Microsomes were isolated from interleukin-1 β -stimulated A549 cells, pre-incubated with the test compounds for 15 min, and PGH₂ ($20 \mu\text{mol}\cdot\text{L}^{-1}$) was added as substrate for mPGES-1. The indole derivative MK-886, a well-recognized inhibitor of mPGES-1 (Claveau *et al.*, 2003; Kojima *et al.*, 2004), was used as a reference compound, and this compound concentration-dependently blocked PGE₂ formation with an $IC_{50} \approx 2 \mu\text{mol}\cdot\text{L}^{-1}$ (Figure 2B). As shown in Figure 2B, MC potently and concentration-dependently suppressed PGE₂ formation with an $IC_{50} = 1 \mu\text{mol}\cdot\text{L}^{-1}$. Variation of the pre-incubation periods (2 to 60 min) did not affect the efficiency of MC (not shown). The MC derivative, S-MC, which has only one syncarpic acid moiety (Figure 1), also inhibited mPGES-1 activity, although less potently ($IC_{50} = 10 \mu\text{mol}\cdot\text{L}^{-1}$, Figure 2B). As the IBP-C (Figure 1) was hardly active (Figure 2B), the syncarpic acid moieties of MC are seemingly important for inhibition of mPGES-1. The polyphenol resveratrol failed to inhibit mPGES-1 up to $10 \mu\text{mol}\cdot\text{L}^{-1}$, and neither indomethacin nor celecoxib ($10 \mu\text{mol}\cdot\text{L}^{-1}$, each) caused suppression of PGE₂ formation in this assay (data not shown).

Myrtucommulone is a reversible and substrate concentration-independent inhibitor of mPGES-1

To investigate if MC blocks mPGES-1 in a reversible manner, microsomal preparations of A549 cells were pre-incubated with MC or MK-886 at 0.3 or $3 \mu\text{mol}\cdot\text{L}^{-1}$ for 15 min, each. The sample containing $3 \mu\text{mol}\cdot\text{L}^{-1}$ inhibitor was split: one aliquot was diluted 10-fold with reaction buffer to obtain a final inhibitor concentration of $0.3 \mu\text{mol}\cdot\text{L}^{-1}$; the other aliquot was not altered. Then, PGH₂ was added to each sample to start

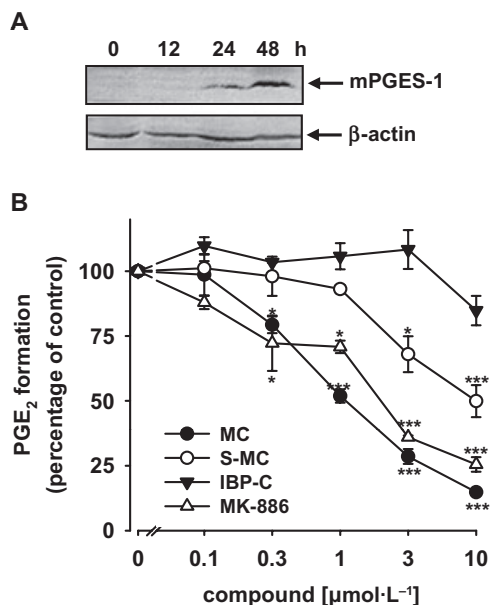


Figure 2 Effects of myrtucommulone (MC) on the activity of microsomal prostaglandin E₂ synthase (mPGES)-1 in a cell-free assay. (A) Expression of mPGES-1 in interleukin-1 β -stimulated A549 cells. Treatment of A549 cells with interleukin-1 β ($1 \text{ ng}\cdot\text{mL}^{-1}$) in culture medium containing 2% fetal calf serum resulted in a time-dependent increase of mPGES-1 (16 kDa) protein. Proteins were subjected to gel electrophoresis on a 14% SDS-PA gel and analysed by Western blotting. β -Actin (45 kDa) was used as loading control. Data are representative of three independent experiments. (B) Microsomal preparations of interleukin-1 β -stimulated A549 cells were pre-incubated with vehicle (dimethyl sulphoxide, DMSO), MK-886, MC, semi-myrtucommulone (S-MC) and the isobutyrophenone-core (IBP-C) for 15 min at 4°C , and the reaction was started by addition of $20 \mu\text{mol}\cdot\text{L}^{-1}$ PGH₂. After 1 min at 4°C the reaction was terminated by using a stop solution containing 11β -PGE₂ (1 nmol) as internal standard. PGE₂ was quantified by RP-HPLC as described in the *Methods* section. Data are given as mean \pm SE, $n = 3$, * $P < 0.05$ or *** $P < 0.001$ vs. vehicle (0.1% DMSO) control, ANOVA + Tukey HSD post hoc tests.

PGE₂ formation. Both MK-886 and MC moderately reduced PGE₂ formation at $0.3 \mu\text{mol}\cdot\text{L}^{-1}$, whereas at $3 \mu\text{mol}\cdot\text{L}^{-1}$, PGE₂ synthesis was potently inhibited (Figure 3A). However, dilution of the latter mixture to yield a final concentration of $0.3 \mu\text{mol}\cdot\text{L}^{-1}$, prior to addition of substrate abolished the inhibitory effect of MK-886 and of MC, implying that both compounds inhibit mPGES-1 in a reversible fashion.

Inhibition of mPGES-1 by MC could be influenced by the concentration of PGH₂ as substrate. However, decreasing the PGH₂ concentration from $20 \mu\text{mol}\cdot\text{L}^{-1}$ to $1 \mu\text{mol}\cdot\text{L}^{-1}$ did not affect the potency of MC for inhibition of mPGES-1, and also not of MK-886 (Figure 3B), which may exclude a substrate-competitive inhibitory mechanism. More detailed studies by using different concentrations of MC at varying substrate concentrations were performed to evaluate the kinetic mechanism for inhibition of mPGES-1. Eadie Hofstee plots indicate a non-competitive type of inhibition (Figure 3C).

Effects of myrtucommulone on the formation of PGE₂ and 6-oxo PGF_{1 α} in intact A549 cells

A549 cells treated with interleukin-1 β for 48 h were shown to strongly express COX-2 and mPGES-1 (Jakobsson *et al.*, 1999),

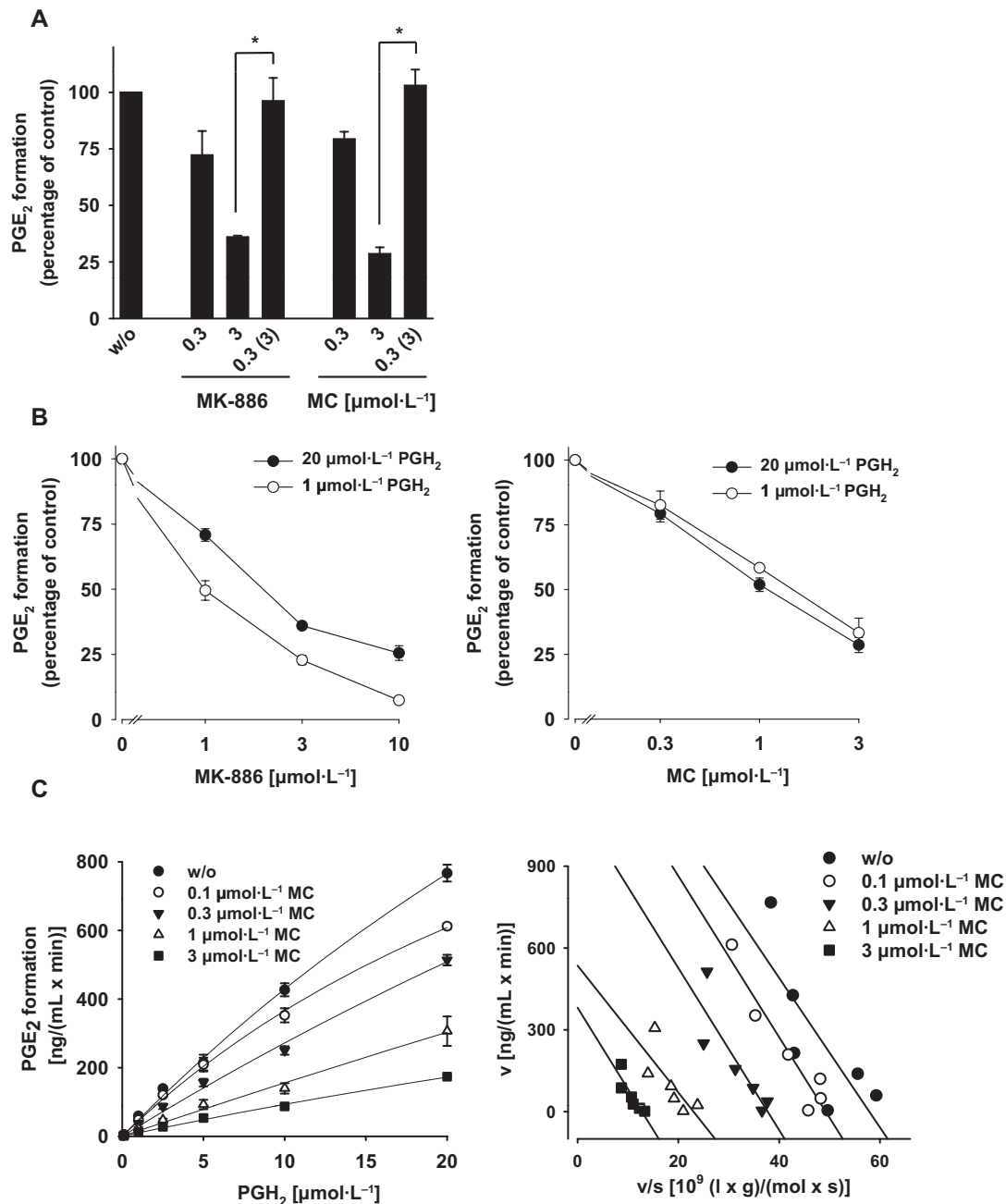


Figure 3 Myrtycommulone (MC) inhibits microsomal prostaglandin E₂ synthase (mPGES)-1 in a reversible and substrate concentration-independent manner. (A) Microsomal preparations of interleukin-1 β -stimulated A549 cells were pre-incubated with 3 $\mu\text{mol}\cdot\text{L}^{-1}$ MK-886 or MC, or with vehicle (dimethyl sulphoxide, DMSO, w/o) for 15 min at 4°C, each. Then, one aliquot of the samples was diluted with assay buffer 10-fold, whereas the other one was not altered, and 20 $\mu\text{mol}\cdot\text{L}^{-1}$ PGH₂ was added to start the reaction. For comparison, microsomal preparations were pre-incubated for 15 min at 4°C with 0.3 $\mu\text{mol}\cdot\text{L}^{-1}$ MK-886 or MC, or with vehicle (DMSO), and then 20 $\mu\text{mol}\cdot\text{L}^{-1}$ PGH₂ was added (no dilution). Then, all samples were incubated for 1 min on ice, and PGE₂ formation was analysed as described. Data are given as mean \pm SE, $n = 3$, * $P < 0.05$, ANOVA + Tukey HSD post hoc tests. (B) The potency of MC for mPGES-1 inhibition was compared at 1 and 20 $\mu\text{mol}\cdot\text{L}^{-1}$ PGH₂ as substrate. In a variation of the general procedure, the amount of PGE₂ was quantified for 1 $\mu\text{mol}\cdot\text{L}^{-1}$ PGH₂ by use of a PGE₂ high sensitivity EIA Kit according to the manufacturer's protocol. Data are given as mean \pm SE, $n = 3-4$. (C) The activity of mPGES-1 was determined at different PGH₂ and different MC concentrations, as indicated (left panel). In a variation of the general procedure, the amount of PGE₂ was quantified by use of a PGE₂ high sensitivity EIA Kit according to the manufacturer's protocol. Data are given as mean \pm SE, $n = 3-4$. Eadie-Hofstee analysis (right panel) was performed by using the original data.

whereas COX-1 was not detected (Asano *et al.*, 1996). Hence, in activated A549 cells, PGH₂ should derive from COX-2, and the conversion to PGE₂ should be catalysed by mPGES-1. However, cPGES and mPGES-2 are still likely to contribute,

although to a lesser extent (Park *et al.*, 2006). A549 cells, treated with interleukin-1 β for 48 h were pre-incubated with MC, MK-886 or vehicle (DMSO) for 10 min prior to stimulation with 2.5 $\mu\text{mol}\cdot\text{L}^{-1}$ A23187 plus 1 $\mu\text{mol}\cdot\text{L}^{-1}$ arachidonic

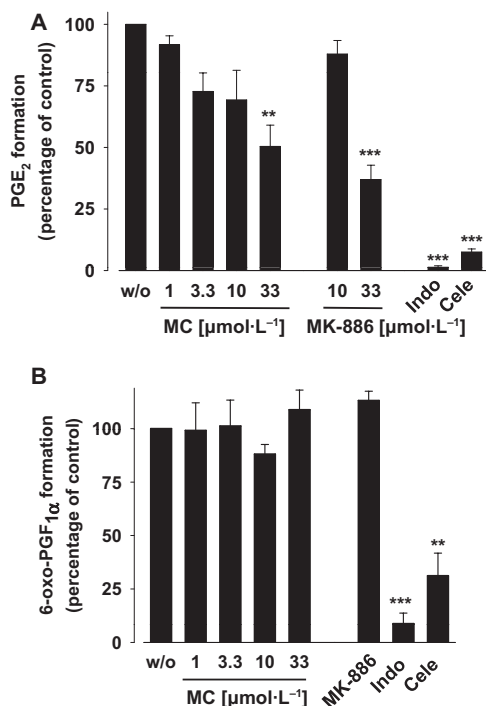


Figure 4 Effects of myrtucommulone (MC) on the formation of prostaglandin (PGE₂) and 6-oxo-PGF_{1α} in intact A549 cells. (A) PGE₂ formation. A549 cells (4×10^6 per-mL) were pre-incubated with MK-886, MC or vehicle (dimethyl sulphoxide, DMSO, w/o) for 10 min, then cellular PGE₂ synthesis was elicited by addition of $2.5 \mu\text{mol}\cdot\text{L}^{-1}$ A23187 plus $1 \mu\text{mol}\cdot\text{L}^{-1}$ arachidonic acid and [³H]arachidonic acid (18.4 kBq). After 15 min at 37°C , formed [³H]PGE₂ was analysed by RP-HPLC and liquid scintillation counting as described in the *Methods* section. (B) 6-oxo-PGF_{1α} formation. Interleukin-1 β -stimulated A549 cells (5×10^6 per-mL) were pre-incubated with the indicated concentrations of MC, with MK-886 ($30 \mu\text{mol}\cdot\text{L}^{-1}$), indomethacin (Indo, $10 \mu\text{mol}\cdot\text{L}^{-1}$), celecoxib (Cele, $5 \mu\text{mol}\cdot\text{L}^{-1}$) or vehicle (DMSO, w/o) for 15 min prior to addition of $30 \mu\text{mol}\cdot\text{L}^{-1}$ arachidonic acid. After 15 min at 37°C the amount of 6-oxo-PGF_{1α} was assessed by enzyme-linked immunosorbent assay as described in the *Methods* section. Data are given as mean \pm SE, $n = 3$, $^{**}P < 0.01$ or $^{***}P < 0.001$ vs. vehicle (0.1% DMSO) control, ANOVA + Tukey HSD post hoc tests.

acid and [³H]arachidonic acid (18.4 kBq). Under these stimulation conditions, cells are activated via a marked elevation of intracellular Ca^{2+} , and the need for the release of endogenous arachidonic acid by cPLA₂ as substrate for COX-2 is circumvented, allowing the exclusion of indirect inhibitory effects of MC. The PGE₂ formed was separated by RP-HPLC and quantified by liquid scintillation counting.

The formation of PGE₂ was concentration-dependently reduced by MC with an apparent IC₅₀ of approximately $30 \mu\text{mol}\cdot\text{L}^{-1}$ (Figure 4A). Notably, a complete inhibition of PGE₂ synthesis could not be achieved, neither by MC nor by MK-886, and compared with the cell-free assay, both compounds were obviously less potent. Thus, at $33 \mu\text{mol}\cdot\text{L}^{-1}$, MK-886 reduced PGE₂ formation only by approximately 60% of control. In contrast to the mPGES-1 inhibitors, indomethacin ($10 \mu\text{mol}\cdot\text{L}^{-1}$) or celecoxib ($5 \mu\text{mol}\cdot\text{L}^{-1}$) essentially abolished PGE₂ formation (Figure 4A). In parallel, the effects of MC and MK-886 on the formation of the PGI₂ degradation product 6-oxo-PGF_{1α} in A549 cells was analysed as a measure of cellular COX-2 activity. In fact, no reduction of 6-oxo-PGF_{1α}

formation was observed for MC (1 to $33 \mu\text{mol}\cdot\text{L}^{-1}$) or for MK-886 ($30 \mu\text{mol}\cdot\text{L}^{-1}$) (Figure 4B), whereas indomethacin ($10 \mu\text{mol}\cdot\text{L}^{-1}$) as well as celecoxib ($5 \mu\text{mol}\cdot\text{L}^{-1}$) reduced 6-oxo-PGF_{1α} formation under these conditions.

Effects of myrtucommulone on the formation of PGE₂ and 12-HHT in human whole blood

Heparinized human whole blood was pre-incubated with MC for 5 min, prior to stimulation with lipopolysaccharide ($10 \mu\text{g}\cdot\text{mL}^{-1}$) for 5 h. For measuring PGE₂, we established a novel assay system consisting of a combination of RP-HPLC and ELISA because separation of PGE₂ by HPLC was necessary prior to immunological detection. In fact, when we assessed PGE₂ levels by ELISA, neither MC nor MK-886 led to reduced PGE₂ levels, instead rather increased PGE₂ signals were obtained (not shown). Possibly, inhibition of mPGES-1 shunts PGH₂ into other metabolic pathways, and these metabolites seemingly interfere with the PGE₂ antibody (cross reactivity) in the ELISA. Such shunting of PGH₂ was recognized also in mPGES-1 KO mice, where enhanced levels of thromboxane (Tx)_{A2}, PGD₂, PGF_{2α} and PGI₂ were detected (Scholich and Geisslinger, 2006). First, the formed PGE₂ was isolated from plasma by solid phase extraction and separated by RP-HPLC. The semi-purified PGE₂ was then collected and quantified by ELISA. As shown in Figure 5A, at $1 \mu\text{mol}\cdot\text{L}^{-1}$ MC PGE₂ synthesis was reduced by $33 \pm 8\%$, but higher concentrations of MC did not decrease the PGE₂ level further ($42 \pm 5\%$ inhibition at $30 \mu\text{mol}\cdot\text{L}^{-1}$). Also, inhibition of mPGES-1 by $30 \mu\text{mol}\cdot\text{L}^{-1}$ MK-886 failed to completely suppress PGE₂ synthesis, thus, $61 \pm 9\%$ PGE₂ still remained, and higher concentrations (up to $100 \mu\text{mol}\cdot\text{L}^{-1}$) caused no further decrease of PGE₂ levels. In contrast, COX inhibition by celecoxib ($20 \mu\text{mol}\cdot\text{L}^{-1}$) or indomethacin ($50 \mu\text{mol}\cdot\text{L}^{-1}$) efficiently reduced PGE₂ formation to $24 \pm 13\%$ and $16 \pm 3\%$ respectively (Figure 5A, left panel). It should be noted that combination of these inhibitors did not increase their effects. Thus, addition of $10 \mu\text{mol}\cdot\text{L}^{-1}$ MC did not significantly improve the potency of MK-886 ($30 \mu\text{mol}\cdot\text{L}^{-1}$) or of celecoxib ($5 \mu\text{mol}\cdot\text{L}^{-1}$, suboptimal concentration) in PGE₂ synthesis (Figure 5A, right panel).

In order to discriminate between the effects of MC on mPGES-1 and on COX enzymes in whole blood, we also determined the influence of MC on the formation of the COX product 12-HHT. The formation of 12-HHT in whole blood (no pretreatment with aspirin and CV4151) after stimulation with Ca^{2+} -inophore and arachidonic acid, mainly deriving from COX-1, was not inhibited by MC (up to $33 \mu\text{mol}\cdot\text{L}^{-1}$), whereas aspirin ($30 \mu\text{mol}\cdot\text{L}^{-1}$) or indomethacin ($20 \mu\text{mol}\cdot\text{L}^{-1}$) potently inhibited 12-HHT synthesis (Figure 5B). These findings indicate that suppression of PGE₂ production by MC in whole blood was not the result of interference with the COX enzymes.

Discussion

The development of selective mPGES-1 inhibitors is of major interest as an alternative strategy to traditional NSAIDs (non-steroidal anti-inflammatory drugs) and coxibs for the treatment of chronic inflammation (Jachak, 2007). The plant

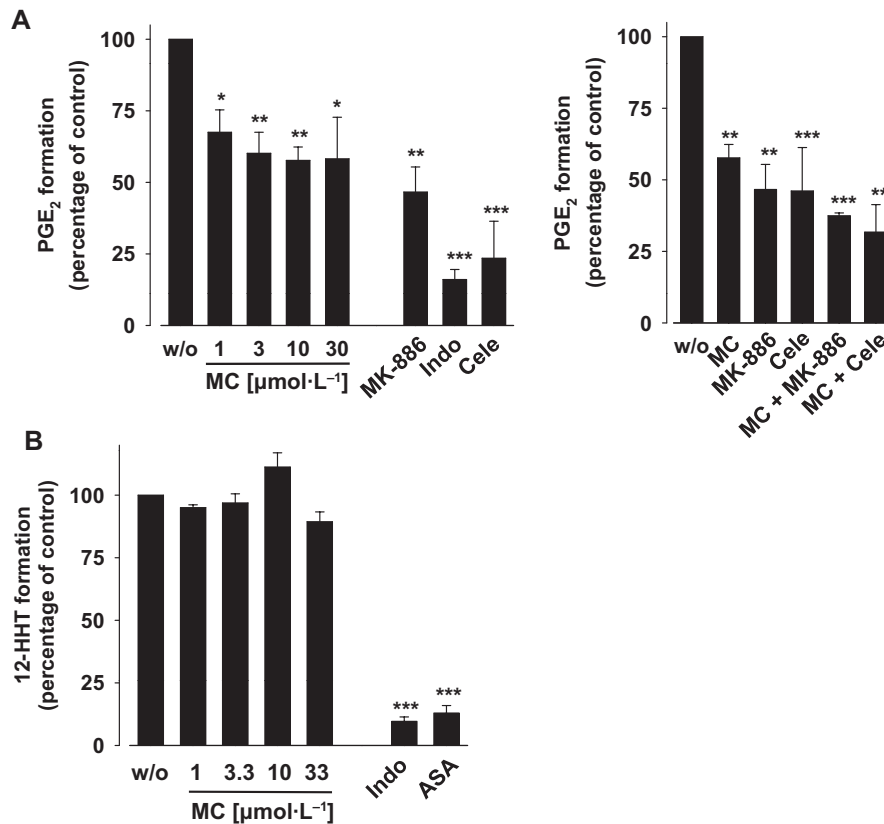


Figure 5 Effects of myrtucommulone (MC) on the formation of prostaglandin (PG)E₂ and 12-HHT (12(S)-hydroxy-5-cis-8,10-trans-heptadecatrienoic acid in human whole blood). (A) PGE₂ formation. Heparinized human whole blood, treated with 1 $\mu\text{mol}\cdot\text{L}^{-1}$ thromboxane synthase inhibitor CV4151 and 50 $\mu\text{mol}\cdot\text{L}^{-1}$ aspirin, was pre-incubated with vehicle (dimethyl sulphoxide, DMSO, w/o), MC (at the indicated concentrations), MK-886 (30 $\mu\text{mol}\cdot\text{L}^{-1}$), indomethacin (Indo, 50 $\mu\text{mol}\cdot\text{L}^{-1}$) and celecoxib (Cele, 20 $\mu\text{mol}\cdot\text{L}^{-1}$ left panel, 5 $\mu\text{mol}\cdot\text{L}^{-1}$ right panel), or with combinations of MC (10 $\mu\text{mol}\cdot\text{L}^{-1}$), MK-886 (30 $\mu\text{mol}\cdot\text{L}^{-1}$) and celecoxib (5 $\mu\text{mol}\cdot\text{L}^{-1}$) as indicated. After 5 min at room temperature, PGE₂ was induced by addition of 10 $\mu\text{g}\cdot\text{mL}^{-1}$ lipopolysaccharide. After 5 h at 37°C, PGE₂ was extracted from plasma by solid phase extraction, separated by RP-HPLC and quantified by enzyme-linked immunosorbent assay as described in the *Methods* section. (B) 12-HHT formation. Heparinized whole blood was pre-incubated with MC or vehicle (DMSO, w/o), at the indicated concentrations for 10 min, and arachidonic acid (100 $\mu\text{mol}\cdot\text{L}^{-1}$) and Ca²⁺-inophore (30 $\mu\text{mol}\cdot\text{L}^{-1}$) were added to induce cyclooxygenase-product formation. After 10 min at 37°C, 12-HHT was extracted from blood plasma by RP-18 solid phase extraction and analysed by RP-HPLC as described in the *Methods* section. Indomethacin (Indo, 20 $\mu\text{mol}\cdot\text{L}^{-1}$) and aspirin (ASA, 30 $\mu\text{mol}\cdot\text{L}^{-1}$) were used as controls. Data are given as mean \pm SE, $n = 3$, * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$ vs. vehicle (0.1% DMSO) control, ANOVA + Tukey HSD post hoc tests.

kingdom is a valuable source for anti-inflammatory substances, and several hundred natural compounds of plant origin have been described to interfere with PG biosynthesis. Investigations aiming to reveal the underlying molecular mechanisms of these active compounds have mainly aimed at COX-1 and 2 or phospholipase A₂ as relevant targets. However, to our knowledge, no natural compound has been described yet that affects a PGES. Here, we demonstrate that MC from myrtle inhibits mPGES-1, leading to suppression of PGE₂ synthesis without significant effects on the COX enzymes.

The functionality of MC as inhibitor of mPGES-1 was demonstrated in this study by applying three different assays. These were: (i) a cell-free assay measuring the direct conversion of PGH₂ to PGE₂ by mPGES-1; (ii) a cellular assay determining PGE₂ formation from exogenously added arachidonic acid; and finally, (iii) a whole blood assay measuring PGE₂ after exposure to lipopolysaccharide. MC reduced the formation of PGE₂ in all these assays at approximately 1 to 3 $\mu\text{mol}\cdot\text{L}^{-1}$. Nevertheless, PGE₂ formation in intact A549 cells

or whole blood was not completely suppressed by MC, even not at high concentrations. A similar pattern was found for the mPGES-1 inhibitor MK-886, whereas the COX-2 selective inhibitor, celecoxib, and the non-selective COX inhibitor, indomethacin, efficiently blocked PGE₂ synthesis in these assays. The basal PGE₂ formation in whole blood, which was not inhibited by MC, is presumably mPGES-1-independent and could also not be reduced by the COX-2-selective celecoxib or by combinations of celecoxib with MC or with MK-886, implying that the remaining PGE₂ formation could occur via COX-1 and other PGES (Park *et al.*, 2006), not affected by MC or MK-886. Intriguingly, interleukin-1 β -treated mouse embryo fibroblasts of mPGES-1 KO mice still produced about 30% of PGE₂ relative to the output in wild type (WT) animals (Kapoor *et al.*, 2006), and the *in vivo* levels of PGE₂ in mPGES-1 KO mice were only reduced by 52%, compared with WT animals (Trebino *et al.*, 2003). Finally, PGE₂ was formed from PGH₂ in the cell-free assay containing heat-inactivated PGES, suggesting non-enzymatic conversion of PGH₂ to PGE₂ (Koeberle A, Werz O, unpubl. data). Conse-

quently, a complete suppression of PGE₂ formation *in vivo* may be achieved by blocking PGH₂ generation (i.e. by COX-1 and 2 inhibition) but apparently not by blockade of mPGES-1 alone. Although this might be detrimental in respect to the potency of mPGES-1 inhibitors, selective inhibition of mPGES-1 without affecting constitutively expressed PGES isoforms is considered to be an advantage in terms of side effects (Samuelsson *et al.*, 2007).

Interestingly, both MC and MK-886 potently inhibit cellular 5-lipoxygenase product formation (Gillard *et al.*, 1989; Feisst *et al.*, 2005). As a consequence, blockade of leukotriene formation and shunting of arachidonic acid into prostanoid biosynthetic pathways might counteract the inhibitory effect of MC and MK-886 on mPGES-1. However, although shunting effects might impair the efficiency of MC and MK-886 in whole blood compared with cell-free assays, they should be negligible in A549 cells, which do not express significant levels of 5-lipoxygenase, as monitored by Western blotting (Brock *et al.*, 2005).

Inhibition of PGE₂ formation was not observed until 3 µmol·L⁻¹ MC in A549 cells, whereas MC was effective at 1 µmol·L⁻¹ in the cell-free test system and in the whole blood assay. Similarly, MK-886 (10 µmol·L⁻¹) only marginally reduced PGE₂ formation in intact A549, despite potent mPGES-1 inhibition in the cell-free assay. These discrepancies in potency might be explained by the different assay conditions. Thus, A23187 rapidly induces substantial PGH₂ synthesis in A549 cells, compared with the slower effects of lipopolysaccharide in whole blood. Possibly, MC (and also MK-886) inhibits the transfer of PGH₂ from COX-2 to mPGES-1 resulting in lower inhibitory efficiency when high amounts of PGH₂ are provided by COX-2. On the other hand, A549 cells express high amounts of multidrug resistance-1 (MDR-1) and lung resistance protein (LRP) (Trussardi *et al.*, 1998), resulting potentially in enhanced cellular efflux of MC.

Mechanistic investigations revealed that MC as well as MK-886 inhibited mPGES-1 in a reversible fashion, not dependent on substrate concentration, and our kinetic data indicated a non-competitive mode of inhibition for MC. Both MC and MK-886 represent lipophilic carboxylic acids and based on their fatty acid-like character, they may bind to the same sites at mPGES-1 as polyunsaturated fatty acids, like arachidonic acid and 15-deoxy-Δ^{12,14}-PGJ₂ that inhibit mPGES-1 (Quraishi *et al.*, 2002). In fact, MK-886 functions as potent leukotriene (LT) synthesis inhibitor [IC₅₀ = 2.5 nmol·L⁻¹, (Gillard *et al.*, 1989)] by competing with arachidonic acid for binding to 5-lipoxygenase-activating protein (FLAP) (Mancini *et al.*, 1993), and also MC potently blocks cellular LT biosynthesis, partially due to direct inhibition of 5-lipoxygenase (Feisst *et al.*, 2005). On the other hand, MC could simply scavenge PGH₂ by a redox mechanism, because PGH₂ is sensitive to reducing agents, causing formation of PGF₂ (Thoren and Jakobsson, 2000; Mancini *et al.*, 2001). However, MC is a weak antioxidant [IC₅₀ up to 162 µmol·L⁻¹, (Rosa *et al.*, 2003)], and also incubation with MC in the test tube caused no significant enhanced degradation of PGH₂ (data not shown). Therefore, a specific interaction between mPGES-1 and MC, depending on defined structural elements, is reasonable.

In addition to the effectiveness of MC to block PGE₂ formation, focus has been placed also on its selectivity

for mPGES-1 as the point of attack. MC did not inhibit the activity of isolated COX-2 and hardly interfered with isolated COX-1. Also, despite significant suppression of PGE₂ formation in cell-based assays, the concomitant generation of 6-oxo PGF_{1α} in A549 cells and 12-HHT in whole blood was not reduced. Hence, interference with COX enzymes is seemingly not the reason for reduced PGE₂ formation by MC in the intact cell assays. Moreover, suppression of PLA₂ enzymes by MC as possible reason for reduced PGE₂ levels can be ruled out, because PGE₂ formation was suppressed in A549 cells in the presence of exogenously added arachidonic acid.

In conclusion, MC is the first natural compound of plant origin reported to inhibit mPGES-1. Moreover, MC advantageously blocks LT formation (Feisst *et al.*, 2005), and suppression of both the LT and the PG biosynthetic pathway would be of advantage over inhibition of a single pathway, not only in terms of anti-inflammatory effectiveness, but also due to a lower incidence of (gastrointestinal) side effects (Celotti and Laufer, 2001). MC might exhibit a similar pharmacological profile as MK-886, because both compounds potently inhibit LT and PGE₂ formation, without interfering with COX isoforms at low concentrations. Unfortunately, no studies have been performed yet that aimed to assess the bioavailability and achievable plasma concentrations of MC, either in animals or in humans. Detailed analysis of MC or MC-containing extracts of myrtle in appropriate animal models of inflammation (our ongoing studies) will give further insights into the pharmacological potential of MC and of myrtle for therapeutic use. Altogether, in regard of its effectiveness in whole blood and its functionality on mPGES-1, but not on COX enzymes, MC has an interesting pharmacological profile as a dual mPGES-1 and 5-lipoxygenase inhibitor.

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Conflict of interest

None.

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