Identification of Molecular Targets of the Oligomeric Nonprenylated Acylphloroglucinols from *Myrtus communis* and Their Implication as Anti-Inflammatory Compounds

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**ABSTRACT**

Myrtucommulone (MC) and semimyrtucommulone (S-MC) are unique oligomeric, nonprenylated acylphloroglucinols contained in the leaves of myrtle (*Myrtus communis*). Although extracts of myrtle have been traditionally used in folk medicine for the treatment of various disorders, studies addressing select cellular or molecular pharmacological properties of these extracts or specific ingredients thereof are rare. Here, we show for the first time that MC and S-MC potently suppress the biosynthesis of eicosanoids by direct inhibiting cyclooxygenase-1 and 5-lipoxygenase in vitro and in vivo at IC_{50} values in the range of 1.8 to 29 μM. Moreover, we show that MC and S-MC prevent the mobilization of Ca^{2+} in polymorphonuclear leukocytes, mediated by G protein signaling pathways at IC_{50} values of 0.55 and 4.5 μM, respectively, and suppress the formation of reactive oxygen species and the release of elastase at comparable concentrations. The isobutyrophenone core of MC as well as S-MC was much less potent or even not active at all. In addition, MC or S-MC only partially inhibited peroxide formation or failed to block Ca^{2+} mobilization and elastase release when polymorphonuclear leukocytes were challenged with ionomycin that circumvents G protein signaling for cell activation. We conclude that, in view of their ability to suppress typical proinflammatory cellular responses, the unique acylphloroglucinols MC and S-MC from myrtle may possess an anti-inflammatory potential, suggesting their therapeutic use for the treatment of diseases related to inflammation and allergy.

Myrtle (*Myrtus communis*, Myrtaceae) is a Mediterranean shrub used as a culinary spice and as a folk medicine, for example, in the treatment of diabetes mellitus and as an antiseptic agent. In fact, myrtle extracts have been traditionally used in folk medicine for the treatment of various disorders, implying potential as medicine for the treatment of diseases related to oxidative stress, including inflammatory disorders. Myrtle contains a great variety of compounds like bitter substances, ethereal oil, and tannic substances (el-Sissi and el-Ansary, 1967). In addition to these rather ubiquitous ingredients that are present in many plants, myrtle contains unique oligomeric, nonprenylated acylphloroglucinols such as myrtucommulone (MC) and semimyrtucommulone (S-MC) (Appendino et al., 2002), which are considered to be responsible for the antioxidative (Rosa et al., 2003) and antibacterial (Appendino et al., 2002) activities of myrtle extracts. Myrtle preparations.

Inflammation is a complex pathophysiological event, mediated and regulated by multiple cells that exert a number of proinflammatory functions, including chemokine and...
cytokine liberation, release of bioactive mediators and proteases, expression of adhesion molecules, and formation of highly reactive molecules [e.g., reactive oxygen species (ROS) or nitric oxide]. Activation of G protein-coupled receptors (GPCRs), present on the surface of inflammatory cells, plays an important role for elicitation of functional cellular responses (Johnson and Druy, 2002). Upon ligation by a specific agonist, GPCRs transduce the signal through different signaling molecules to elevate the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)), one key event for the subsequent recruitment of effector enzymes such as 5-lipoxygenase (5-LO) or cyclooxygenases (COXs) that produce leukotrienes (LTs) or prostaglandins (PGs) and thromboxanes from arachidonic acid (AA), respectively (Funk, 2001). Suppression of LT and PG synthesis by interfering with the 5-LO and COX pathways represents an efficient pharmacological approach for the treatment of inflammatory diseases (Funk, 2001). In addition, elevation of [Ca\(^{2+}\)]\(_i\), causes release of proteases (e.g., leukocyte elastase or cathepsin G) and formation of ROS (Krause et al., 1990; Parekh and Penner, 1997), both which destroy invading particles but also damage cells and tissues of the host.

We and others have previously shown that the prenylated acylphloroglucinol hyperforin from Hypericum perforatum exerts anti-inflammatory properties in various cellular test systems (Albert et al., 2002; Heilmann et al., 2003; Feist and Werz, 2004). For myrtle and its nonprenylated acylphloroglucinols, information concerning anti-inflammatory properties is substantially still lacking. In the present study, we examined the anti-inflammatory potential of the acylphloroglucinols MC and S-MC that share an isobutyrophenone core (IBP-C) decorated with two (MC) and one (S-MC) syncarboxylic acid moieties. We identified MC and to a lesser extent also S-MC as direct inhibitors of 5-LO and COX-1, being able to suppress the biosynthesis of PGs and LTs in vitro and in vivo. In addition, these nonprenylated acylphloroglucinols inhibit the release of elastase and the formation of ROS, apparently by their ability to block receptor-coupled Ca\(^{2+}\) mobilization.

**Materials and Methods**

**Materials.** MC and S-MC were isolated from myrtle leaves as described previously (Appendino et al., 2002). The IBP-C was synthesized from S-MC (G. Appendino, unpublished data). The compounds were dissolved in dimethyl sulfoxide (DMSO) and kept in the dark at −20°C, and freezing/thawing cycles were kept to a minimum.

Materials used were Nycoprep (PAA Laboratories GmbH, Coelbe, Germany); ionomycin, AA, MeO-Suc-Ala-Ala-Pro-Val-p-nitroanilide, cytochalasin B, and fMLP, N-formyl-methionyl-leucyl-phenylalanine (fMLP) (Sigma-Aldrich, St. Louis, MO); 13\((\text{Z})\)-hydroxy-5,8,11-cis-13-trans-eicosatetraenoic acid per 10\(^6\) cells. 5-LO product formation is expressed as nanograms of 5-LO products per 10\(^6\) cells, which include LTB\(_4\) and its all-trans-isomers, 5(S,12(S)-di-hydroxy-6,10-trans-8,14-cis-eicosatetraenoic acid and 5(S)-hydroxy-6-trans-8,11,14-cis-eicosatetraenoic acid. Cysteinyl LTs (LTC\(_4\), D\(_{12}\), and E\(_{12}\)) were not detected, and oxidation products of LTB\(_{12}\) were not determined.

Determination of 12-Lipoxygenase and Cyclooxygenase-1 Product Formation in Platelets. Intact platelets (1 × 10\(^8\), resuspended in 1 ml of PGC buffer) were preincubated for 10 min with each of the test compounds at room temperature, and 10 \(\mu\)M AA was added. After 10 min at 37°C, the incubation was stopped with the addition of 1 ml of methanol and 30 \(\mu\)l of 1 N HCl, and 200 ng of prostaglandin B\(_1\) and 500 \(\mu\)l of PBS were added. Formed AA metabolites were extracted and analyzed by HPLC as described (Werz et al., 2002).

For determination of COX-1 activity in cell homogenates, platelets (1 × 10\(^8\)) were resuspended in 1 ml of PBS (containing 1 mM EDTA) and cooled on ice for 5 min. After sonication (3 × 5 s), the test compounds were added (5–10 min at 4°C), the samples were preincubated for 30 s at 37°C and CaCl\(_2\) and AA (2 mM and 10 \(\mu\)M, respectively) were added to start the 5-LO reaction. After 10 min, the incubation was terminated and 5-LO product formation was determined as described for the intact cells.

Determination of 12-Lipoxygenase and Cyclooxygenase-1 Product Formation in Platelets. Intact platelets (1 × 10\(^8\), resuspended in 1 ml of PGC buffer) were preincubated for 10 min with each of the test compounds at room temperature, and 10 \(\mu\)M AA was added. After 10 min at 37°C, the incubation was stopped with the addition of 1 ml of methanol and 30 \(\mu\)l of 1 N HCl, and 200 ng of prostaglandin B\(_1\) and 500 \(\mu\)l of PBS were added. After centrifugation (10 min, 800g), the 12-LO product 12\((\text{Z})\)-hydroxy-5,8-cis-10-trans-14-cis-eicosatetraenoic acid and the COX-1-derived AA metabolite 12\((\text{Z})\)-hydroxy-5-cis-8,10-trans-heptadecatrienoic acid (12-HHT) were extracted using C-18 solid phase extraction columns and analyzed by HPLC as described (Albert et al., 2002).

**Expression and Purification of 5-LO from Escherichia coli.** Expression of 5-LO, performed in E. coli JM 109 cells transfected with pT3–5-LO, and purification of 5-LO by affinity chromatography (Sigma A2767) was performed as described previously (Fischer et al., 2003). Partially purified 5-LO was immediately used for in vitro activity assays.

**Determination of Cyclooxygenase-2 Product (6-Keto PGF\(_{1\alpha}\)) Formation in Mono Mac 6 Cells.** Mono Mac 6 (MM6) cells were grown with or without transforming growth factor β and calcitriol for 96 h as described previously (Brungs et al., 1995). Six hours before harvest, LPS (100 ng/ml) was added. Cells were harvested, washed twice, resuspended in PGC buffer (5 × 10\(^6\) cells/ml),
and incubated with AA (30 μM) for 15 min at 37°C. After centrifugation (300g, 5 min, 4°C), the amount of 6-keto PGF1α, released was assessed by ELISA using a monoclonal antibody against 6-keto PGF1α as described elsewhere (Albert et al., 2002).

**Determination of Cellular Peroxide Formation.** Measurement of peroxides in PMNLs was conducted using the peroxide-sensitive fluorescence dye DCF-DA, which reacts with hydrogen peroxide but also with nitric oxide (Rao et al., 1992). Freshly isolated PMNLs (1 × 10^6 in 1 ml of PGC buffer) were preincubated with test compounds or vehicle (DMSO, control) for 10 min at rt in the dark and then treated with DCF-DA (1 μg/ml) for 1 min at 37°C before the addition of the stimuli. The fluorescence emission at 530 nm was measured after excitation at 480 nm in a thermally controlled (37°C) fluorometer cuvette with continuous stirring in a spectrofluorometer (Aminco-Bowman series 2; AMINCO-Bowman SPF, Silver Spring, MD). The mean fluorescence data measured 5 min after stimulus addition are expressed as arbitrary fluorescence units.

**Determination of Leukocyte Elastase Release.** PMNLs (5 × 10^6), resuspended in 1 ml of PGC buffer, were preincubated with the test compounds or vehicle (DMSO, control) for 10 min at rt in the dark. For stimulation with 1 μM fMLP, cells were preincubated with cytochalasin B (10 μM) for 5 min at 37°C and ionomycin was used as stimulus without pretreatment with cytochalasin B. The reaction was terminated after 10 min at 37°C by placing the samples on ice for 2 min. After centrifugation (1000g, 5 min, 4°C), the supernatants were incubated with MeO-Suc-Ala-Ala-Pro-Val-p-nitroanilide (200 μM) for 5 min at 37°C. The extent of p-nitrophenol released was measured at 405 nm.

**Measurement of Intracellular Ca^{2+} Levels.** PMNLs (1 × 10^6/ml PGC buffer) were incubated with 2 μM Fura-2/AM for 30 min at 37°C, washed, resuspended in 1 ml of PG buffer, and preincubated with the test compounds or vehicle (DMSO, control) for 10 min at rt in the dark. Cells were then transferred into a thermally controlled (37°C) fluorometer cuvette in a spectrofluorometer (Aminco-Bowman series 2) with continuous stirring, and stimuli were added. The fluorescence emission at 510 nm was measured after excitation at 340 and 380 nm, respectively. Intracellular Ca^{2+} levels were calculated according to the method of Grynkiewicz et al. (1985). \( F_{\text{max}} \) (maximal fluorescence) was obtained by lysing the cells with 1% Triton X-100 and \( F_{\text{min}} \) by chelating Ca^{2+} with 10 mM EDTA.

**Statistics.** The statistic program GraphPad PRISM 3.0 (GraphPad Software Inc., San Diego, CA) was used for statistical comparisons. Statistical evaluation of the data was performed using Student’s t test for unpaired observations. \( P < 0.05 \) was considered significant.

**Results**

**Effects of Myrtucommulone, Semimyrtucommulone, and the Isobutyrophenone Core on COX Activity.** Freshly isolated human platelets preincubated with MC, S-MC, IBP-C, and the Isobutyrophenone Core (Fig. 2A), or vehicle (DMSO) for 10 min were stimulated with 10 μM AA, and the formation of the COX-1-derived product 12-HHT was analyzed. MC suppressed 12-HHT formation with an IC_{50} of 17 μM (Fig. 2A), whereas S-MC was somewhat less potent (IC_{50} = 29 μM). Of interest, the IBP-C blocked COX-1 most efficiently (IC_{50} = 5 μM). To determine whether the compounds exert their inhibitory effects by direct interference with the COX-1 enzyme, inhibition of 12-HHT formation was assessed in platelet homogenates. As can be seen from Fig. 2B, MC, S-MC, and IBP-C suppressed COX-1 activity in the cell-free assay with comparable potencies (IC_{50} values are 6, 30, and 6.5 μM, respectively) as found for intact cell assays, implying that the compounds directly interfere with COX-1 activity. No inhibition of 12-LO product synthesis was observed (data not shown). To determine the effects of MC on the activity of COX-2, LPS-stimulated MM6 cells were used as selective source for COX-2 (Albert et al., 2002). In contrast to COX-1 from platelets, MC (up to 30 μM) failed to significantly block the activity of the inducible COX-2 in MM6 cells (data not shown).

**Effects of Myrtucommulone, Semimyrtucommulone, and the Isobutyrophenone Core on 5-LO Activity.** Inhibition of 5-LO activity was determined in freshly isolated human PMNLs or for isolated human recombinant 5-LO enzyme by assessment of the formation of the 5-LO-derived products LTB_{4}, its all-trans-isomers, and 5(S)-hydro(pero)xy-6-trans-8,11,14-cis-eicosatetraenoic acid. For investigation of cellular 5-LO product formation, MC, S-MC, IBP-C, or vehicle (DMSO) was added to PMNLs; after 10 min, cells were stimulated with 1 μM ionomycin plus 20 μM AA. As shown in Fig. 3A, 5-LO product synthesis was dose-dependently reduced by all compounds. MC was most efficient (IC_{50} = 1.8 μM), whereas S-MC and the IBP-C showed equal potency (IC_{50} = 7 μM). In contrast, the activity of the closely related (eosinophilic) 15-LO was not significantly affected by the compounds up to 30 μM (data not shown).

To confirm a direct inhibition of 5-LO enzyme, human recombinant 5-LO was expressed in E. coli and partially purified and the effects of MC, S-MC, and IBP-C were determined. At a substrate concentration of 10 μM AA, MC and S-MC suppressed 5-LO product formation with an IC_{50} of approximately 5 and 8 μM, respectively (Fig. 3B). The IBP-C showed lower potency and the IC_{50} value was determined at approximately 26 μM. Moreover, the potency of MC was assessed at various AA concentrations (3, 10, and 30 μM). The effectiveness of MC to suppress 5-LO activity was not impaired by elevating the substrate concentration. Instead, the potency slightly increased at higher amounts of AA (Fig. 3C). This suggests that AA does not compete with MC for binding at a common binding cleft of 5-LO but rather points to an uncompetitive mode of action.
the absence of inhibitors was 176.3 fMLP (100 nM). The IC50 values were determined at 0.55 and 0.24 M, respectively (Fig. 5). In contrast, the IBP-C up to 30 µM was hardly active. Of interest, peroxide production evoked by ionomycin that circumvents GPCR signaling was inhibited by MC with an IC50 value of 0.8 µM. However, also at higher concentrations (30 µM) of MC, some peroxide formation still remained (approximately 25% of control) and S-MC failed to completely block the ionomycin-induced response (Fig. 6).

**Effects of Myrtucommulone and Semimyrtucommulone on the Formation of Reactive Oxygen Species.** Freshly isolated human PMNLs were used to determine the effects of the test compounds on agonist-induced ROS formation. After preincubation with MC, S-MC, IBP-C, or vehicle (DMSO) for 10 min, the amounts of peroxides released upon stimulation were determined by measuring the fluorescence of the peroxide-sensitive dye DCF-DA after oxidation. Peroxide formation evoked by stimulation with FMLP (1 M) was concentration-dependently suppressed by MC (IC50 = 0.24 µM) and S-MC (IC50 = 1.9 µM) (Fig. 6). In contrast, the IBP-C up to 30 µM was hardly active. Of interest, peroxide production evoked by ionomycin that circumvents GPCR signaling was inhibited by MC with an IC50 value of 0.8 µM. However, also at higher concentrations (30 µM) of MC, some peroxide formation still remained (approximately 25% of control) and S-MC failed to completely block the ionomycin-induced response (Fig. 6).

**Discussion**

Our results suggest an anti-inflammatory potential for myrtle. This suggestion is based on the observation that its acylphloroglucinol constituents MC and S-MC (0.12 and 0.14% dry weight, respectively) suppress various functional cellular leukocyte responses, including the biosynthesis of PGs and LTs, the release of leukocyte elastase, and the formation of peroxides that are of relevance for initiation and maintenance of inflammatory processes. With respect to the molecular mode of action, we show that MC and S-MC potently inhibit the agonist-induced elevation of [Ca2+]i, a determinant in mediating cellular functional responses upon extracellular stimulation. To our knowledge, this is also the first study that identifies molecular targets (5-LO and COX-1) for MC and S-MC.

5-LO and COX-1/2 are the key enzymes in the formation of proinflammatory LTs and PGs, respectively, which play pivotal roles in inflammation and allergy (Funk, 2001). Such
pathophysiological implications can be visualized by 5-LO or COX gene knock-out studies as well as by experimental and clinical application of 5-LO and/or COX inhibitors and receptor antagonists against the eicosanoids (Funk, 2001). Numerous potent and selective COX inhibitors are on the market and have been frequently used for intervention with pain, fever, and inflammatory disorders for many years (DeWitt, 1999; Celotti and Laufer, 2001). In contrast, no 5-LO inhibitor is presently available for clinical therapy, despite the strong need for such drugs for intervention with asthma and allergic rhinitis, rheumatoid arthritis, and cardiovascular diseases (Werz and Steinhiber, 2005), probably because of the inefficacy and the lack of safety and selectivity of 5-LO inhibitors observed in animal models and clinical trials. Hence, the identification and development of suitable pharmacological remedies in this respect are of great interest.

MC and S-MC and also the IBP-C suppressed COX-1 activity in intact cells and in a cell-free assay with similar potencies each. Hence, COX-1 inhibition is not a unique effect of the oligomeric nonprenylated acylphloroglucinols MC and S-MC but rather seems to be related to the acylphloroglucinol moiety present in all three structures. Nevertheless, COX-1 was markedly and concentration-dependently suppressed by the compounds investigated with comparable potencies to aspirin (Schror, 1997) and therefore implies therapeutic potential for the treatment of COX-1-related disorders. Of interest, the inducible isoenzyme COX-2 from LPS-treated MM6 cells was not significantly inhibited by MC, which agrees with the fact that the acylphloroglucinol hyperforin from *H. perforatum* blocks COX-1 from platelets but not COX-2 from monocytes (Albert et al., 2002).

In addition, MC and S-MC also act as potent 5-LO inhibi-
tors, effective in intact cells as well as on the partially purified 5-LO with similar potencies. In contrast to COX-inhibition, the IBP-C was less potent for 5-LO and MC showed 6-fold superior effectiveness over S-MC in intact cells. Among 5-LO inhibitors that can be categorized into redox-active, iron-chelating, and nonredox-type inhibitors, most compounds act by reducing the iron in the active site, keeping it in the inactive ferrous state (Werz, 2002). In fact, different extracts from myrtle as well as MC and S-MC were shown to possess antioxidant activity in different experimental conditions (Rosa et al., 2003; Romani et al., 2004). In contrast to 5-LO inhibition, S-MC was much more powerful than MC in protecting oxidation of linoleic acid in vitro and lipid peroxidation in homogenates (Rosa et al., 2003), indicating that the efficacy of S-MC to scavenge hydroxyl and peroxy radicals is superior over that of MC. Moreover, the antioxidant effects of MC were observed at much higher concentrations (IC50 values between 13.6 and 162 μM; Rosa et al., 2003) compared with those required to suppress 5-LO activity (IC50 = 1.8 to approximately 10 μM), indicating that mechanisms other than simply reducing or unspecific scavenging properties cause 5-LO inhibition. It should be noted that the closely related 12- and 15-LOs were not inhibited by MC or S-MC, again excluding unspecific uncoupling of LO redox cycles because of reducing properties of the compounds. On the other hand, variations of the AA concentration did not alter

Fig. 4. Myrtucommunolone, semimyrtucommunolone, and the isobutyrophenone core inhibit receptor-coupled Ca2+ mobilization in PMNLs. Freshly isolated PMNLs (107/ml PGC buffer) were loaded with 2 μM Fura-2/AM. Cells were preincubated in the absence (control) or presence of MC, S-MC, and the IBP-C at the indicated concentrations for 10 min at rt and stimulated with 100 nM fMLP or 1 μM ionomycin at 37°C. The fluorescence was measured, and [Ca2+]i was calculated as described. Results are expressed as the percentage of the Ca2+ level (mean ± S.E.; n = 3) versus control (100%). Stimulation with fMLP or ionomycin elevated [Ca2+]i from 80.5 ± 7.8 to 244.4 ± 19.8 nM or to 443.4 ± 53.4 nM, respectively.

Fig. 5. Effects of myrtucommunolone, semimyrtucommunolone, and the isobutyrophenone core on agonist-induced elastase release from PMNLs. Freshly isolated PMNLs (107 in 1 ml of PGC buffer) were preincubated with MC, S-MC, and the IBP-C core for 10 min at rt. For stimulation with 1 μM fMLP, cells were preincubated with cytochalasin B (10 μM) for 5 min. Ionomycin (1 μM) was used as stimulus without pretreatment with cytochalasin B. After 10 min at 37°C, cells were put on ice for 10 min and centrifuged, and the supernatants were assayed for elastase activity as described. Data are given as mean ± S.E. (n = 3) and are expressed as percentage of the control (stimulated cells without inhibitor, 100%).
5-LO inhibition by MC, suggesting that the compound may act in a noncompetitive manner. Recently, we showed that hyperforin selectively inhibits 5-LO in vivo and in vitro in an uncompetitive yet far unknown fashion without affecting other LO activities (Albert et al., 2002). Because of their common acylphloroglucinol moiety, MC and S-MC may act in the same manner and at the same site as hyperforin; elucidation of this site is a future task.

Besides inhibition of eicosanoid biosynthesis, MC and S-MC, but not the IBP-C, significantly suppressed the increase in [Ca\(^{2+}\)]\(_i\), evoked by the GPCR ligand fMLP, and in analogy to inhibition of COX-1 and 5-LO, MC was approximately 8-fold more potent compared with S-MC. Many effector enzymes such as phospholipases and protein kinases respond to and are regulated by an elevation of [Ca\(^{2+}\)]\(_i\), leading to functional cellular responses like degranulation and ROS formation (Mandeville and Maxfield, 1996). In accordance with their ability to suppress Ca\(^{2+}\) mobilization, MC and S-MC markedly blocked the release of leukocyte elastase and the formation of peroxides elicited by fMLP in established and optimized activity assay at similar concentrations needed to block the fMLP-induced increase in [Ca\(^{2+}\)]\(_i\). Note that despite the different stimulation conditions (i.e., different fMLP concentration, inclusion of cytochalasin B) used to evoke ROS formation, elastase release, and Ca\(^{2+}\) mobilization, the IC\(_{50}\) values of MC (0.24–0.9 \(\mu\)M) or S-MC (1.9–4.5 \(\mu\)M) for all these responses are quite similar. We conclude that suppression of Ca\(^{2+}\) mobilization is the major cause for inhibition of fMLP-induced peroxide formation and elastase release by MC and S-MC. This hypothesis is favored also by the fact that inhibition of responses induced by ionomycin that circumvents G protein signaling pathways supported by the antioxidant properties observed by others (Rosa et al., 2003; Romani et al., 2004).

In summary, we have shown that the unique oligomeric, nonprenylated acylphloroglucinols MC and, to a lesser extent, S-MC compromise important cellular responses of inflammatory cells, suggesting a therapeutic potential for myrtle preparations in the treatment of inflammatory and allergic diseases. Further studies using methods to assess an anti-inflammatory action of myrtle and MC or S-MC in animals must remain to be conducted to allow the judgment of the potential for the treatment of inflammation in humans.

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References


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