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Identification of molecular targets of the oligomeric non-prenylated acylphloroglucinols from *Myrtus communis* and their implication as anti-inflammatory compounds*

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Running title: Antiinflammatory properties of myrtle compounds

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Abbreviations used: AA, arachidonic acid; COX, cyclooxygenase; DCF-DA, 2´,7´-dichlorofluorescein diacetate; fMLP, N-formyl-methionyl-leucyl-phenylalanine; GPCR, G protein-coupled receptor; 12-HHT, 12(S)-hydroxy-5-cis-8,10-trans-heptadecatrienoic acid; 5-H(P)ETE, 5(S)-hydro(pero)xy-6-trans-8,11,14-cis-eicosatetraenoic acid; 12-H(P)ETE, 12(S)-hydro(pero)xy-5,8-cis-10-trans-14-cis-eicosatetraenoic acid; 15-H(P)ETE, 15(S)-hydro(pero)xy-5,8,11-cis-13-trans-eicosatetraenoic acid; 13(S)-HPODE, 13(S)-hydroperoxy-9Z,11E-octadecadienoic acid; IBP-C, isobutyrophenone core; LO, lipoxygenase; LT, leukotriene; MC, myrtucommulone; MM6, Mono Mac 6; PBS, phosphate buffered-saline pH 7.4; PG, prostaglandin; PGC buffer, PBS containing 1 mg/ml glucose and 1 mM $CaCl_2$; PLC, phospholipase C; PMNL, polymorphonuclear leukocytes; ROS, reactive oxygen species; S-MC, semi-myrtucommulone.

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Abstract

Myrtucommulone (MC) and semi-myrtucommulone (S-MC) are unique, oligomeric, non-prenylated acylphloroglucinols contained in the leaves of myrtle (*Myrtus communis* L.). 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 signalling pathways at IC_{50} values of 0.55 and 4.5 μM, respectively, and suppress the formation of reactive oxygen species (ROS) and the release of elastase at comparable concentrations. The isobutyrophenone core of MC and S-MC was much less potent or even not active at all. Also, MC or S-MC only partially inhibited peroxide formation or failed to block Ca^{2+} mobilization and elastase release when PMNL were challenged with ionomycin that circumvents G protein signalling 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.

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Introduction

Myrtle (*Myrtus communis* L., 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 reported to possess anti-hyperglycaemic (Elfellah et al., 1984; Sepici et al., 2004; Onal et al., 2005), antibacterial (Al-Saimary et al., 2002; Bonjar, 2004) and analgesic (Levesque and Lafont, 2000) properties. Recent reports have described antioxidant activities of different extracts of myrtle and certain ingredients thereof (Rosa et al., 2003; Hayder et al., 2004; Romani et al., 2004), 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). Aside of these rather ubiquitous ingredients that are present in many plants, myrtle contains unique, oligomeric, non-prenylated acylphloroglucinols such as myrtucommulone (MC) and semi-myrtucommulone (S-MC) (Appendino et al., 2002) which are considered to be responsible for the antioxidative (Rosa et al., 2003) and the antibacterial (Appendino et al., 2002) activities of myrtle preparations.

Inflammation is a complex pathophysiological event, mediated and regulated by multiple cells that exert a number of select 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 NO). Activation of G protein-coupled receptors (GPCRs), present on the surface of inflammatory cells, play an important role for elicitation of functional cellular responses (Johnson and Druey, 2002). Upon ligation by a specific agonist, GPCRs transduce the signal through different signalling 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 (COX) that produce leukotrienes (LTs) or prostaglandins (PGs) and thromboxanes from arachidonic acid (AA), respectively (Funk, 2001). Suppression of LT and

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PG synthesis by interfering with the 5-LO and COX pathways represent 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; Feisst and Werz, 2004). For myrtle and its non-prenylated 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) syncarpic 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 non-prenylated 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 before (Appendino et al., 2002). The IBP-C was synthesized from S-MC (Appendino, G., unpublished results). The compounds were dissolved in dimethylsulfoxide (DMSO) and kept in the dark at – 20°C, and freezing thawing cycles were kept to a minimum.

Materials used: Nycoprep, PAA Laboratories; ionomycin, AA, MeO-Suc-Ala-Ala-Pro-Val-p-nitroanilide, cytochalasin B, and N-formyl-methionyl-leucyl-phenylalanine (fMLP), Sigma; 13(S)-hydroperoxy-9Z,11E-octadecadienoic acid (13(S)-HPODE), Cayman; Fura-2/AM, Alexis; 2´, 7´-dichlorofluorescein diacetate (DCF-DA), Molecular Probes.

Cells

Human platelets and PMNL were freshly isolated from leukocyte concentrates obtained at St Markus Hospital (Frankfurt, Germany). In brief, venous blood was taken from healthy adult donors and leukocyte concentrates were prepared by centrifugation at 4000 × g/20 min/20°C. PMNL were immediately isolated by dextran sedimentation, centrifugation on Nycoprep cushions (PAA Laboratories), and hypotonic lysis of erythrocytes as described previously (Werz et al., 2002). PMNL (7.5×10^6 cells /ml; purity > 96-97%) were finally resuspended in PBS containing 1 mg/ml glucose and 1 mM $CaCl_2$ (PGC buffer) as indicated. Investigation of cell viability by trypan blue exclusion using freshly isolated PMNL indicated no significant toxic effects of MC, S-MC and the IBP-C during preincubation periods up to 30 min.

For isolation of platelets, platelet-rich plasma, obtained from supernatants (800 × g, 10 min, rt) after centrifugation of leukocyte concentrates on Nycoprep cushions, was mixed with PBS pH 5.9 (3:2, vol/vol), centrifuged (2,000 × g, 15 min, rt) and the pelleted platelets were resuspended in PBS pH 5.9 / 0.9 % NaCl (1:1, vol/vol), washed by centrifugation (2,000 × g, 10 min, rt) and finally resuspended in PBS pH 5.9.

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Determination of 5- and 15-lipoxygenase products in PMNL

To assay 5- and 15-LO product formation in intact cells, 7.5×10^6 freshly isolated PMNL were finally resuspended in 1 ml PGC buffer. After preincubation with the test compounds for 10 min at 37°C, the reaction was started by addition of 1 µM ionomycin plus 20 µM AA. After 10 min at 37°C, the reaction was stopped with 1 ml of methanol and 30 µl of 1 N HCl, and 200 ng prostaglandin B_1 and 500 µl of PBS were added. Formed AA metabolites were extracted and analyzed by HPLC as described (Werz et al., 2002).

To determine product formation of purified 5-LO enzyme, 5-LO protein (0.1 µg in 10 µl) was added to 990 µl PBS, containing 1 mM EDTA and 1 mM ATP on ice and the indicated compounds were added. After 5-10 min on ice, the samples were preincubated for 30 sec at 37°C and $CaCl_2$ and AA (2 mM and 10 µM, 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.

15-LO product formation is expressed as ng 15(S)-hydro(pero)xy-5,8,11-cis-13-trans-eicosatetraenoic acid (15-H(P)ETE) per 10^6 cells. 5-LO product formation is expressed as ng 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)-hydro(peroxy)-6-trans-8,11,14-cis-eicosatetraenoic acid (5-H(P)ETE). Cysteinyl LTs (LTC_4, D_4 and E_4) were not detected and oxidation products of LTB_4 were not determined.

Determination of 12-lipoxygenase and cyclooxygenase-1 product formation in platelets

Intact platelets (1×10^8, resuspended in 1 ml PGC buffer) were preincubated for 10 min with the test compounds at room temperature and 10 µM AA was added. After 10 min at 37°C, incubations were stopped by addition of 1 ml of methanol and 30 µl of 1 N HCl, 200 ng of prostaglandin B1 (internal standard) and 500 µl of PBS were added. After centrifugation (10

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min, 800 × g), the 12-LO product 12(S)-hydro(pero)xy-5,8-cis-10-trans-14-cis-eicosatetraenoic acid (12-H(P)ETE) and the COX-1-derived AA metabolite 12(S)-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).

For determination of COX-1 activity in cell homogenates, platelets (1×10^8) were resuspended in 1 ml PBS (containing 1 mM EDTA) and cooled on ice for 5 min. After sonication (3×5 sec), the test compounds were added (5-10 min at 4°C), the samples were pre-incubated for 30 sec at 37°C and the incubation was started by the addition of Ca^{2+} and AA (2 mM and 10 µM final concentrations, respectively). After 10 min at 37°C, the incubation was stopped with 1 ml of methanol and the formed 12-H(P)ETE and 12-HHT were extracted and analyzed as described for intact cells.

Expression and purification of 5-LO from E. coli.

Expression of 5-LO, performed in *E. coli* JM 109 cells transfected with pT3-5LO, and purification of 5-LO by ATP 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 (MM)6 cells were grown with or without transforming growth factor beta and calcitriol for 96 hrs as described (Brungs et al., 1995). Six hrs prior 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 (300 × g, 5 min, 4°C), the amount of 6-keto $PGF_{1\alpha}$ released was assessed by ELISA using a monoclonal antibody against 6-keto $PGF_{1\alpha}$ as described elsewhere (Albert et al., 2002).

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Determination of cellular peroxide formation

Measurement of peroxides in PMNL was conducted using the peroxide-sensitive fluorescence dye DCF-DA, that reacts with hydrogen peroxide, but also with nitric oxide (Rao et al., 1992). Freshly isolated PMNL (1×10^7 in 1 ml 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 prior addition of the stimuli. The fluorescence emission at 530 nm was measured after excitation at 480 nm in a thermally controlled (37°C) fluorimeter cuvette with continuous stirring in a spectrofluorometer (Aminco-Bowman series 2). The mean fluorescence data measured 5 min after stimulus addition are expressed as arbitrary fluorescence units.

Determination of leukocyte elastase release

PMNL (5×10^7), resuspended in 1 ml 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, ionomycin was used as stimulus without pre-treatment with cytochalasin B. The reaction was terminated after 10 min at 37°C by placing the samples on ice for 2 min. After centrifugation (1,000 × g, 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

PMNL (1×10^7/ml PGC buffer) were incubated with 2 µM Fura-2/AM for 30 min at 37°C, washed, resuspended in 1 ml PGC buffer and preincubated with the test compounds or vehicle

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(DMSO (control)) for 10 min at rt in the dark. Then, cells were transferred into a thermally controlled (37°C) fluorimeter 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_{max} (maximal fluorescence) was obtained by lysing the cells with 1% Triton-X 100 and F_{min} by chelating Ca^{2+} with 10 mM EDTA.

Statistics

The statistic program "GraphPad PRISM 3.0" was used for statistical comparisons. Statistical evaluation of the data was performed using Student´s *t* test for unpaired observations. A *P* value of < 0.05 was considered significant.

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Results

Effects of myrtucommulone, semi-myrtucommulone and the isobutyrophenone core on COX activity

Freshly isolated human platelets preincubated with MC, S-MC, IBP-C (chemical structures see **fig. 1**), 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). In order to determine if 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 the 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 (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 (not shown).

Effects of myrtucommulone, semi-myrtucommulone and the isobutyrophenone core on 5-LO activity

Inhibition of 5-LO activity was determined in freshly isolated human PMNL 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-H(P)ETE. For investigation of cellular 5-LO product formation MC, S-MC, IBP-C, or vehicle (DMSO) were added to PMNL and 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

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(IC_{50} = 1.8 µM), whereas S-MC and the IBP-C showed equal potency (IC_{50} ≈ 10 µM). In contrast, the activity of the closely related (eosinophilic) 15-LO was not significantly affected by the compounds up to 30 µM (not shown).

To confirm a direct inhibition of 5-LO enzyme, human recombinant 5-LO was expressed in E. coli, partially purified and the effects of MC, S-MC and the 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 approx. 5 µM and 8 µM, respectively (**fig. 3B**). The IBP-C was less potent and the IC_{50} value was determined at approx. 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.

Myrtucommulone and semi-myrtucommulone suppress G protein-mediated increases in intracellular Ca^{2+}

Elevation of $[Ca^{2+}]_i$ upon cell stimulation by various agonists is a pivotal signal transduction event leading to functional leukocyte responses including degranulation and formation of ROS (Simchowitz and Spilberg, 1979; Krause et al., 1990; Mandeville and Maxfield, 1996). MC as well as S-MC concentration-dependently suppressed the increase in $[Ca^{2+}]_i$ in PMNL evoked by the natural occurring agonist fMLP (100 nM). The IC_{50} values were determined at 0.55 µM and 4.5 µM, respectively (**fig. 4**). The IBP-C was hardly efficient and the IC_{50} was > 30 µM. In control experiments, ionomycin that circumvents G protein signalling was used to induce elevation of $[Ca^{2+}]_i$. Of interest, neither MC nor S-MC (up to 30 µM) could prevent the ionomycin-induced increases in $[Ca^{2+}]_i$, suggesting that the compounds attenuate Ca^{2+}

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mobilization presumably by abrogating G protein signalling or by inhibiting Ca^{2+} fluxes through interference with certain Ca^{2+} channels.

Effects of myrtucommulone and semi-myrtucommulone on the release of leukocyte elastase

Upon agonist challenge, PMNL are capable to release proteases (i.e leukocyte elastase) from intracellular granules, mediated by elevated levels of $[Ca^{2+}]_i$ (Khalfi et al., 1996). The release of elastase from freshly isolated PMNL was assessed by determination of elastase-specific peptide cleavage in supernatants of stimulated cells. After preincubation (10 min) with MC, S-MC, IBP-C, or vehicle (DMSO), cells were challenged with 1 µM fMLP plus 10 µM cytochalasin B or with 1 µM ionomycin for 5 min and elastase activity was analyzed. MC and S-MC, but not the IBP-C, concentration-dependently suppressed degranulation induced by fMLP with IC_{50} values of 0.9 µM and 3.8 µM, respectively (**fig. 5**). In contrast when PMNL were challenged with ionomycin, MC and S-MC (up to 10 µM, each) did not impair elastase release (**fig. 5**).

Effects of myrtucommulone and semi-myrtucommulone on the formation of reactive oxygen species

Freshly isolated human PMNL 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 µM) was concentration-dependently suppressed by MC (IC_{50} = 0.24 µM) and S-MC (IC_{50} = 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 signalling was inhibited by MC with an IC_{50} value of 0.8 µM. However,

also at higher concentrations (30 µM) of MC, some peroxide formation still remained (approx. 25 % of control) and S-MC failed to completely block the ionomycin-induced response (**fig. 6**).

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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 which 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 $[Ca^{2+}]_i$, 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 are 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 Steinhilber, 2005). The inefficacy and the lack of safety and selectivity of 5-LO inhibitors observed in animal models and clinical trials are likely reasons therefore. Hence, the identification and development of suitable pharmacological remedies in this respect is of great interest.

MC and S-MC but 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, non-prenylated acylphloroglucinols MC and S-MC but rather seems to be related

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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 acylphoroglucinol 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 inhibitors, effective in intact cells as well as on the partially purified 5-LO with similar potencies. In contrast to COX-inhibition, for 5-LO the IBP-C was less potent, 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 peroxyl radicals is superior over that of MC. Moreover, the antioxidant effects of MC were observed at much higher concentrations (IC_{50} values between 13.6 to 162 µM (Rosa et al., 2003)) as compared to those required to suppress 5-LO activity (IC_{50} = 1.8 to approx. 10 µM), indicating that other mechanisms 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 due to reducing properties of the compounds. On the other hand, variations of the AA concentration did not alter 5-LO inhibition by MC suggesting that the compound may act in a non-competitive manner. Recently, we showed that hyperforin

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selectively inhibits 5-LO *in vivo* and *in vitro* in an uncompetitive, thus far unknown fashion without affecting other LO activities (Albert et al., 2002). Due to 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 about 8-fold more potent as compared to S-MC. Many effector enzymes like 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 µM) or S-MC (1.9 – 4.5 µM) 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 favoured also by the fact that inhibition of responses induced by ionomycin that circumvents G protein signalling pathways for elevation of $[Ca^{2+}]_i$ either required substantially higher concentrations of MC or S-MC or was hardly evident. Along these lines, we recently found that hyperforin blocked G protein-mediated Ca^{2+} mobilization connected with reduced elastase and peroxide release from PMNL in the same manner as the putative PLC inhibitor U-73122 (Feisst and Werz, 2004) that was shown to possess anti-inflammatory actions in various experimental animal models (Hou et al., 2004). It should be noted that the efficacy of MC and S-MC was most potent for inhibition of peroxide formation, and besides induction by

fMLP, MC was able to partially inhibit also the ionomycin effect in this respect, indicating that the compounds (in particular MC) may also directly interfere with peroxide-producing enzymes and/or 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, non-prenylated 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 in order to allow the judgement of the potential for the treatment of inflammation in humans.

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References

Albert D, Zündorf, I., Dingermann, T., Müller, W.E., Steinhilber, D., and Werz, O. (2002) Hyperforin is a dual inhibitor of 5-lipoxygenase and cyclooxygenase-1. *Biochem Pharmacol* **64**:1767-1775.

Al-Saimary IE, Bakr SS, Jaffar T, Al-Saimary AE, Salim H and Al-Muosawi R (2002) Effects of some plant extracts and antibiotics on Pseudomonas aeruginosa isolated from various burn cases. *Saudi Med J* **23**:802-805.

Appendino G, Bianchi F, Minassi A, Sterner O, Ballero M and Gibbons S (2002) Oligomeric acylphloroglucinols from myrtle (Myrtus communis). *J Nat Prod* **65**:334-338.

Bonjar GH (2004) Antibacterial screening of plants used in Iranian folkloric medicine. *Fitoterapia* **75**:231-235.

Brungs M, Rådmark O, Samuelsson B and Steinhilber D (1995) Sequential induction of 5-lipoxygenase gene expression and activity in Mono Mac 6 cells by transforming growth factor-beta and 1,25-dihydroxyvitamin D3. *Proc. Natl. Acad. Sci. USA* **92**:107-111.

Celotti F and Laufer S (2001) Anti-inflammatory drugs: new multitarget compounds to face an old problem. The dual inhibition concept. *Pharmacol Res* **43**:429-436.

DeWitt DL (1999) Cox-2-selective inhibitors: the new super aspirins. *Mol Pharmacol* **55**:625-631.

Elfellah MS, Akhter MH and Khan MT (1984) Anti-hyperglycaemic effect of an extract of Myrtus communis in streptozotocin-induced diabetes in mice. *J Ethnopharmacol* **11**:275-281.

el-Sissi HI and el-Ansary MA (1967) Tannins and polyphenolics of the leaves of Myrtus communis. *Planta Med* **15**:41-51.

Feisst C and Werz O (2004) Suppression of receptor-mediated Ca2+ mobilization and functional leukocyte responses by hyperforin. *Biochem Pharmacol* **67**:1531-1539.

Fischer L, Szellas D, Radmark O, Steinhilber D and Werz O (2003) Phosphorylation- and stimulus-dependent inhibition of cellular 5-lipoxygenase activity by nonredox-type inhibitors. *Faseb J* **17**:949-951.

Funk CD (2001) Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science* **294**:1871-1875.

Grynkiewicz G, Poenie M and Tsien RY (1985) A new generation of Ca2+ indicators with greatly improved fluorescence properties. *J Biol Chem* **260**:3440-3450.

Hayder N, Abdelwahed A, Kilani S, Ammar RB, Mahmoud A, Ghedira K and Chekir-Ghedira L (2004) Anti-genotoxic and free-radical scavenging activities of extracts from (Tunisian) Myrtus communis. *Mutat Res* **564**:89-95.

Heilmann J, Winkelmann K and Sticher O (2003) Studies on the antioxidative activity of phloroglucinol derivatives isolated from hypericum species. *Planta Med* **69**:202-206.

Hou C, Kirchner T, Singer M, Matheis M, Argentieri D and Cavender D (2004) In vivo activity of a phospholipase C inhibitor, 1-(6-((17beta-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole -2,5-dione (U73122), in acute and chronic inflammatory reactions. *J Pharmacol Exp Ther* **309**:697-704.

Johnson EN and Druey KM (2002) Heterotrimeric G protein signaling: role in asthma and allergic inflammation. *J Allergy Clin Immunol* **109**:592-602.

Khalfi F, Gressier B, Brunet C, Dine T, Luyckx M, Cazin M and Cazin JC (1996) Involvement of the extracellular calcium in the release of elastase and the human neutrophils oxidative burst. *Cell Mol Biol (Noisy-le-grand)* **42**:1211-1218.

Krause KH, Campbell KP, Welsh MJ and Lew DP (1990) The calcium signal and neutrophil activation. *Clin Biochem* **23**:159-166.

Levesque H and Lafont O (2000) [Aspirin throughout the ages: a historical review]. *Rev Med Interne* **21 Suppl 1**:8s-17s.

Mandeville JT and Maxfield FR (1996) Calcium and signal transduction in granulocytes. *Curr Opin Hematol* **3**:63-70.

Onal S, Timur S, Okutucu B and Zihnioglu F (2005) Inhibition of alpha-glucosidase by aqueous extracts of some potent antidiabetic medicinal herbs. *Prep Biochem Biotechnol* **35**:29-36.

Parekh AB and Penner R (1997) Store depletion and calcium influx. *Physiol Rev* **77**:901-930.

Rao KM, Padmanabhan J, Kilby DL, Cohen HJ, Currie MS and Weinberg JB (1992) Flow cytometric analysis of nitric oxide production in human neutrophils using dichlorofluorescein diacetate in the presence of a calmodulin inhibitor. *J Leukoc Biol* **51**:496-500.

Romani A, Coinu R, Carta S, Pinelli P, Galardi C, Vincieri FF and Franconi F (2004) Evaluation of antioxidant effect of different extracts of Myrtus communis L. *Free Radic Res* **38**:97-103.

Rosa A, Deiana M, Casu V, Corona G, Appendino G, Bianchi F, Ballero M and Dessi MA (2003) Antioxidant activity of oligomeric acylphloroglucinols from Myrtus communis L. *Free Radic Res* **37**:1013-1019.

Schror K (1997) Aspirin and platelets: the antiplatelet action of aspirin and its role in thrombosis treatment and prophylaxis. *Semin Thromb Hemost* **23**:349-356.

Sepici A, Gurbuz I, Cevik C and Yesilada E (2004) Hypoglycaemic effects of myrtle oil in normal and alloxan-diabetic rabbits. *J Ethnopharmacol* **93**:311-318.

Simchowitz L and Spilberg I (1979) Generation of superoxide radicals by human peripheral neutrophils activated by chemotactic factor. Evidence for the role of calcium. *J Lab Clin Med* **93**:583-593.

Werz O (2002) 5-Lipoxygenase: Cellular biology and molecular pharmacology. *Cur Drug Target – Inflamm Allergy* **1**:23-44.

Werz O, Burkert E, Samuelsson B, Radmark O and Steinhilber D (2002) Activation of 5-lipoxygenase by cell stress is calcium independent in human polymorphonuclear leukocytes. *Blood* **99**:1044-1052.

Werz O and Steinhilber D (2005) Pharmacological intervention with 5-lipoxygenase: new insights and novel compounds. *Expert Opin Ther Patents* **15**:505-519.

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Legends for Figures

Fig. 1 Chemical structure of myrtucommulone (MC), semi-myrtucommulone (S-MC) and the isobutyrophenone core (IBP-C).

Fig. 2 Effects of myrtucommulone, semi-myrtucommulone and the isobutyrophenone core on COX-1 product formation in human platelets and corresponding homogenates. (**A**) Intact platelets. Freshly isolated human platelets (1×10^8 cells/ml PGC buffer) were preincubated with the indicated concentrations of MC, S-MC and the IBP-C core for 10 min at rt. Then, cells were stimulated with 10 µM exogenous AA, incubated for another 10 min at 37°C and 12-HHT formation was determined by HPLC as described in the Materials and Methods section. The control value (100 %) in absence of inhibitors was 176.3 ± 21.5 ng/10^8 cells. (**B**) Platelet homogenates. Freshly isolated human platelets (1×10^8 cells/ml) were sonicated in PBS containing 1 mM EDTA on ice. MC, S-MC and IBP-C were added, and after 5-10 min on ice, samples were preincubated at 37°C for 30 sec. After addition of $CaCl_2$ and AA (2 mM and 10 µM, respectively) samples were incubated for another 10 min at 37°C and 12-HHT formation was determined by HPLC as described. The control value (100 %) in absence of inhibitors was 62.6 ± 9.4 ng/10^8 cells. Results are given as mean + S.E., n = 3.

Fig. 3 Inhibition of 5-LO product formation by myrtucommulone, semi-myrtucommulone and the isobutyrophenone core. (**A**) Freshly isolated human PMNL (7.5×10^6 in 1 ml PGC buffer) were preincubated with the indicated amounts of MC, S-MC and the IBP-C for 10 min at rt. After addition of 1 µM ionomycin plus 20 µM AA, samples were incubated for another 10 min at 37°C and 5-LO products were determined by HPLC. Results are given as mean + S.E., n = 3-4. The control value (100 %) in absence of inhibitors was 157.8 ± 30.2 ng/10^6 cells.

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(**B**) 5-LO (0.1 µg/ml), expressed in *E. coli,* was purified as described and preincubated with the indicated amounts of MC, S-MC and the IBP-C in 1 ml PG buffer plus 1 mM EDTA for 5-10 min on ice. Samples were prewarmed for 30 sec at 37°C and $CaCl_2$ and AA (2 mM and 10 µM, respectively) were added. After 10 min at 37°C, 5-LO product formation was determined as described. Results are given as mean + S.E., n = 3-4.

(**C**) Purified 5-LO (0.1 µg/ml) was preincubated with MC for 5-10 min on ice. Samples were preincubated for 30 sec at 37°C and 2 mM $CaCl_2$ and AA at the indicated concentrations were added. After 10 min at 37°C, 5-LO product formation was determined as described. Results are given as mean + S.E., n = 3-4.

Fig. 4 Myrtucommulone, semi-myrtucommulone and the isobutyrophenone core inhibit receptor-coupled Ca^{2+} mobilization in PMNL.

Freshly isolated PMNL (10^7/ml PGC buffer) were loaded with 2 µM Fura-2/AM. Cells were preincubated in the absence (control) or presence of MC (**A**), S-MC (**B**) and the IBP-C (**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 $[Ca^{2+}]_i$ was calculated as described. Results are expressed as the percentage of the Ca^{2+} level (mean + SE, n = 3) versus control (100 %). Stimulation with fMLP or ionomycin elevated $[Ca^{2+}]_i$ from 80.5 ± 7.8 nM to 244.4 ± 19.8 nM or to 443.4 ± 53.4 nM, respectively.

Fig. 5 Effects of myrtucommulone, semi-myrtucommulone and the isobutyrophenone core on agonist-induced elastase release from PMNL.

Freshly isolated PMNL (10^7 in 1 ml PGC buffer) were preincubated with MC (**A**), S-MC (**B**) and the IBP-C (**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 pre-treatment with cytochalasin B. After 10 min at 37°C, cells were put on ice for 10

min, 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 %).

Fig. 6 Effects of myrtucommulone, semi-myrtucommulone and the isobutyrophenone core on the formation of peroxides in PMNL.

Freshly isolated PMNL (10^7 in 1 ml PGC buffer) were preincubated with the indicated concentrations of MC (**A**), S-MC (**B**) and the IBP-C (**C**) for 10 min at rt prior addition of DCF-DA (1 µg/ml) for 1 min. Then, fMLP (1 µM) or ionomycin (1 µM) were added and the generation of ROS was measured as described. Data (mean + S.E., n = 3) determined 5 min after addition of stimuli are expressed as percentage of the positive control (100 %). The increase in fluorescence of unstimulated, fMLP-challenged, and ionomycin-treated cells was 89.2 ± 15, 487.8 ± 84.4, and 1071 ± 141 arbitrary fluorescence units, respectively.

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Fig. 1

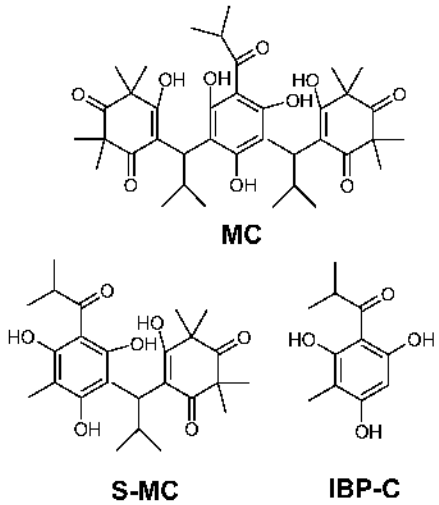

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Fig. 2

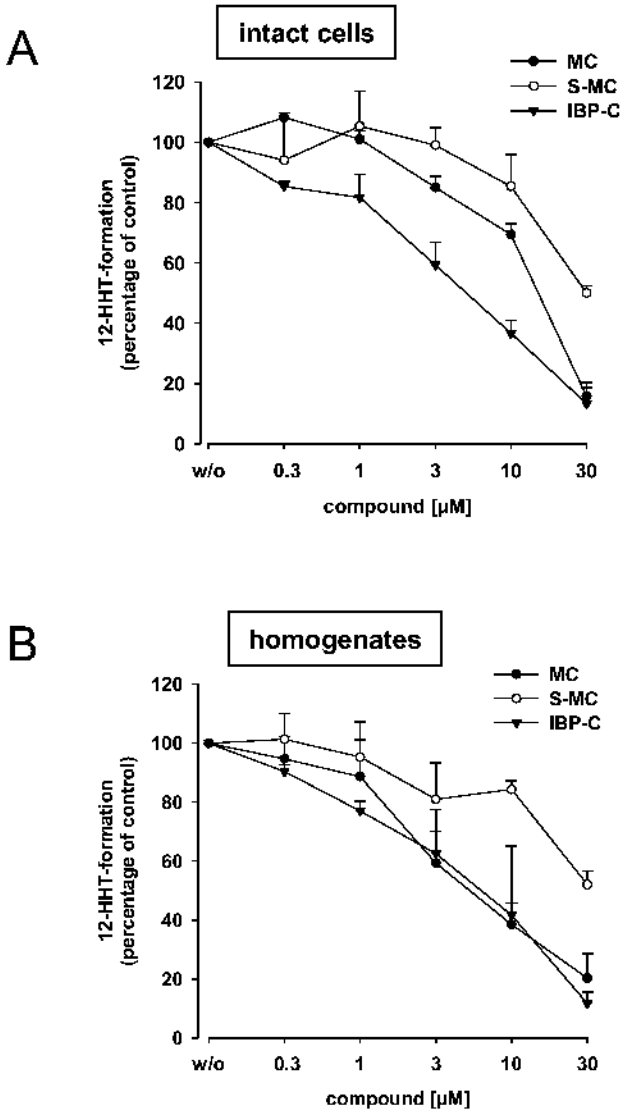

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Fig. 3

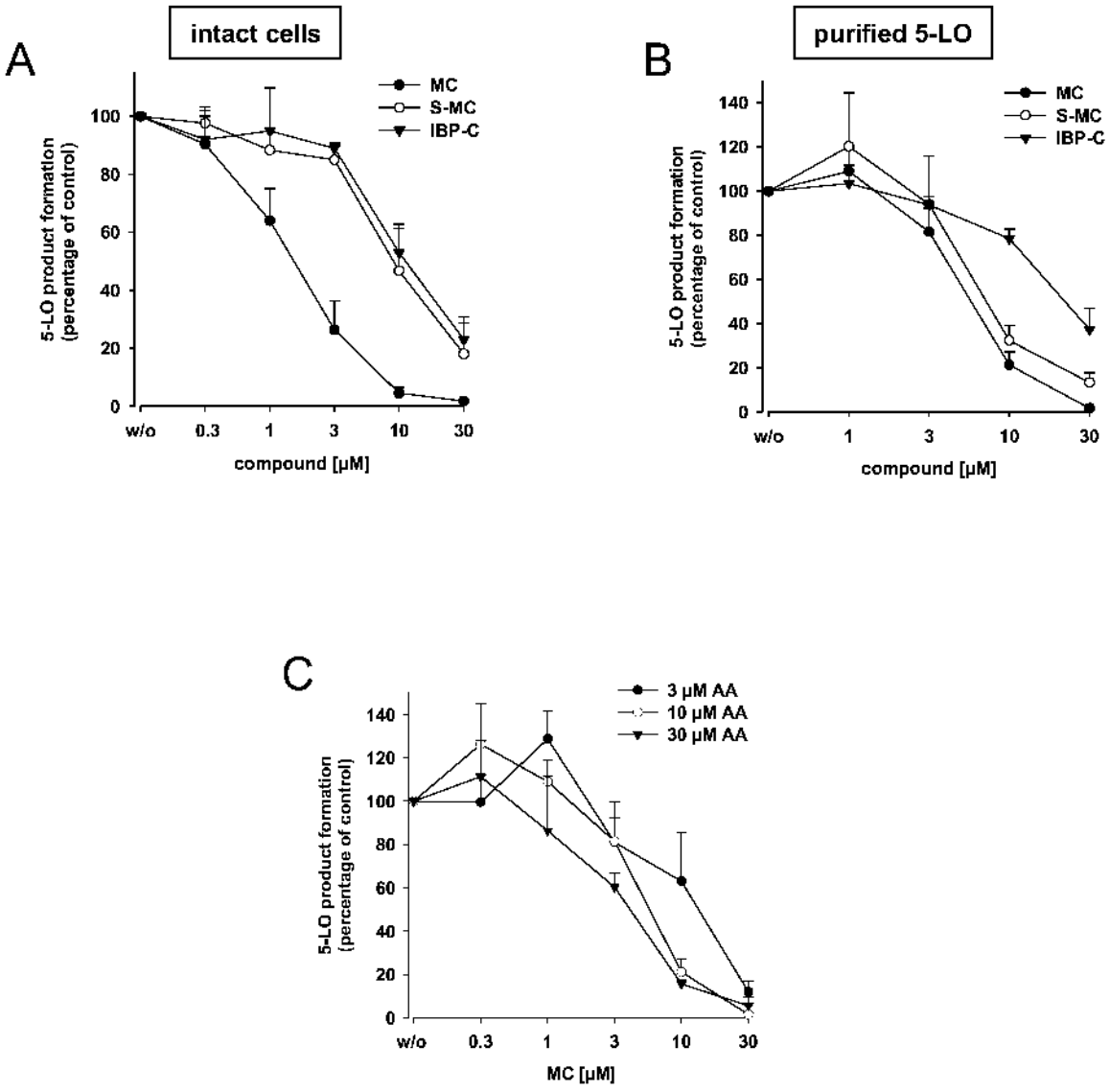

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Fig. 4

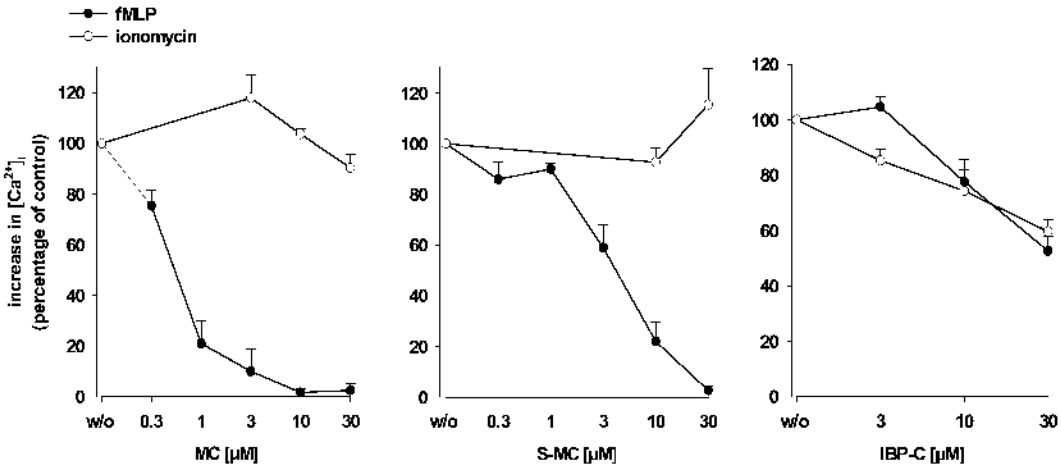

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Fig. 5

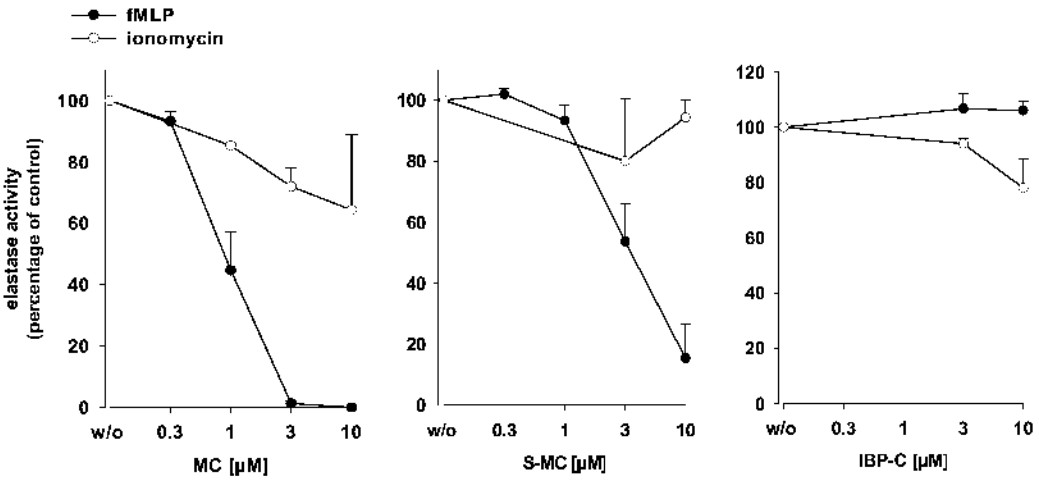

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Fig. 6