

Differential inhibition of inflammatory effector functions by petasin, isopetasin and neopetasin in human eosinophils

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Summary

Background Priming of eosinophils with granulocyte-macrophage colony-stimulating factor (GM-CSF) and subsequent stimulation with platelet-activating factor (PAF) or the anaphylatoxin C5a is associated with a rapid production of leukotrienes (LTs) and release of eosinophil cationic protein (ECP).

Objective This study was designed to determine the effects of the sesquiterpene esters petasin, isopetasin and neopetasin on LT generation and ECP release in eosinophils *in vitro*.

Methods The model of eosinophil activation described above was used to induce LT production and ECP release. Cells were incubated with petasins and control inhibitors prior to priming and stimulation. To analyse intracellular steps of eosinophil activation and determine potential drug targets, some key signalling events were studied. Activity of cytosolic phospholipase A₂ (cPLA₂) was measured by analysing the generation of arachidonic acid (AA). Translocation of 5-lipoxygenase (5-LO) was observed using immunofluorescence microscopy. Intracellular calcium concentrations [Ca²⁺]_i were measured by a bulk spectrofluorometric assay.

Results Whereas all three compounds inhibited LT synthesis, ECP release from eosinophils was blocked by petasin only, but not isopetasin or neopetasin. Similarly, PAF- or C5a-induced increases in [Ca²⁺]_i were completely abrogated by petasin only, whereas isopetasin and neopetasin had significant lower blocking efficacy. Moreover, only petasin, but not isopetasin or neopetasin, prevented increases in cPLA₂ activity and 5-LO translocation from the cytosolic compartment to the nucleus envelope in calcium ionophore-stimulated eosinophils.

Conclusion These data suggest that different petasins may at least partially block different intracellular signalling molecules. To reduce LT synthesis, isopetasin and neopetasin may act at the level of or distal to 5-LO. In contrast, petasin may inhibit inflammatory effector functions in human eosinophils by disrupting signalling events at the level of or proximal to phospholipase C_β (PLC_β), besides its potential inhibitory activity within mitogen-activated protein kinase (MAPK) and LT pathways.

Keywords: cellular activation, eosinophils, inflammation, inflammatory mediators, signal transduction, petasin, isopetasin, neopetasin, zileuton

Clinical and Experimental Allergy, Vol. 31, pp. 1310–1320. Submitted 24 October 2000; revised 21 February 2001; accepted 2 March 2001.

Introduction

Eosinophils are important effector cells, which accumulate at inflammatory sites of many allergic disorders [1–3]. Eosinophil activation appears to be crucial for the

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development of symptoms, since the presence of eosinophils alone does not cause an inflammatory response [4]. There have been a number of *in vitro* studies describing eosinophil activation mechanisms. Hematopoietins, such as IL-3, IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF), increase functional responses of eosinophils to various agonists, including lipid mediators, complement factors or chemokines [5–8]. This effect of haematopoietins, called ‘priming’, is also observed in other granulocyte subtypes [9]. At least under non-adherent conditions, priming of eosinophils appears to be required for both leukotriene (LT) synthesis [6] and degranulation [10].

Lipid mediators (e.g. platelet-activating factor, PAF) and complement factors (e.g. C5a) bind to G-protein-coupled receptors and activate phospholipase C_β (PLC_β) [11], which catalyses hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) to inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 -mediated increases in cytosolic free $[Ca^{2+}]_i$ is essential for the translocation of $cPLA_2$ from the cytosol to the nuclear membrane [12,13]. At the nuclear membrane, cytosolic phospholipase A_2 ($cPLA_2$) liberates arachidonic acid (AA) from phospholipids [14]. To generate LTs, free AA is transferred to 5-lipoxygenase-activating protein (FLAP), which is also located in the nuclear envelope. As shown by immunofluorescence [15,16] and immunoblot analysis [17,18], translocation of 5-lipoxygenase (5-LO) from cytosol to FLAP on membrane compartments appears to be $[Ca^{2+}]_i$ -dependent and essential for the generation of LTA_4 from AA.

In human neutrophils and monocytes, LTA_4 is predominantly converted into LTB_4 by LTA_4 hydrolase [19]. In human eosinophils, LTA_4 is conjugated with reduced glutathione by LTC_4 synthase to form LTC_4 [20]. The cysteinyl-LTs LTC_4 , LTD_4 and LTE_4 increase microvascular permeability and mucus secretion, and promote the contraction of smooth muscle, contributing to bronchoconstriction in asthma [21]. New synthetic drugs have been developed that prevent LT actions, such as 5-LO inhibitors (e.g. zileuton), cysteinyl-LT receptor antagonists (e.g. montelukast) and FLAP inhibitors (e.g. MK-0591) [22].

Alternative medicine approaches are causing increasing interest as possible treatments of many chronic diseases, including asthma and other allergic diseases [23]. In particular, plant extracts appear to have real pharmacological effects. For instance, a Chinese herbal medicine formula has recently been shown to suppress T-helper 2 cytokine expression without affecting IFN- γ expression in a mouse model of allergic asthma [24]. In this article, we analyse the effects of different chemically defined petasin stereoisomers, which are components of a plant extract of *Petasites hybridus* (common name: butterbur) [25], on signal transduction pathways and eosinophil effector

functions, such as generation of cysteinyl-LTs and eosinophil cationic protein (ECP) release. Extracts from leaves and rhizomes of *Petasites hybridus* were used to treat spasms of the gastrointestinal tract and asthma due to its spasmolytic activities in the late middle age [26]. Earlier *in vitro* studies suggested that the plant extracts may block LT synthesis in macrophages [27] and whole leucocyte populations [28].

Materials and methods

Reagents

Ficoll separating solution was from Biochrom KG (Berlin, Germany). GM-CSF was a kind gift from Dr T. Hartung (University of Konstanz, Konstanz, Germany). PAF was obtained from Calbiochem (Lucerne, Switzerland). [5,6,8,9,11,12,14,15- ^3H]-arachidonic acid (1.0 mCi/mL, 1.5 μ g/mL) was from Hartmann Analytic (Braunschweig, Germany). Arachidonyltrifluoromethyl ketone (TFMK) was from Biomol Research Laboratories (Plymouth Meeting, PA, USA). Calcium ionophore A23187 and recombinant human complement factor C5a were purchased from Sigma Chemicals (Buchs, Switzerland). Fura-2-AM and ionomycin were from Roche Diagnostics (Rotkreuz, Switzerland). Zileuton was a kind gift from Abbott (Baar, Switzerland). Montelukast was a kind gift from Merck Sharp Dohme-Chibret (Glattbrugg, Switzerland), Ortho Permeafix[®] was from Ortho Diagnostic Systems (Raritan, NJ, USA). The high pressure carbon dioxide (CO_2) extract of *Petasites hybridus* (Ze339), its isolated active compounds petasin, neopetasin and isopetasin (= PIN) (Fig. 1), and the inactive apolar fraction composed of mainly polyunsaturated fatty acids (FAT), were provided by Max Zeller Söhne AG (Analytical Department, Romanshorn,

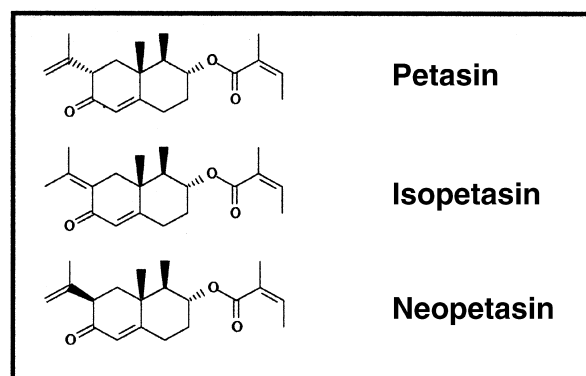


Fig. 1. Structures of the three main active compounds of the Ze339 extract of *Petasites hybridus*. The sesquiterpene esters petasin, isopetasin and neopetasin are isomers with a molecular weight of 316.2.

Switzerland). Anti-5-LO mAb (IgG₁) was obtained from Transduction Laboratories (Lexington, KY, USA). Control monoclonal IgG₁ mAb was from Dako (Zug, Switzerland). CyTM3-conjugated F(ab')₂ fragment donkey anti-mouse IgG (H + L) was from Jackson ImmunoResearch Laboratories (West Grove, PA, USA) and FITC-labelled anti-mouse IgG₁ mAb was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-CD16 mAb microbeads were purchased from Milteny Biotec (Bergisch-Gladbach, Germany). Unless stated otherwise, all other reagents were from Sigma.

Purification of eosinophils

Peripheral blood eosinophils were mostly isolated by negative selection from patients with atopic dermatitis or asthma as previously described [29,30]. In some experiments, eosinophils were also isolated from control individuals with less than 5% eosinophils in peripheral blood.

Stimulation of eosinophils

Unless stated otherwise, purified eosinophils were cultured at $1 \times 10^6/\text{mL}$ in complete culture medium (RPMI-1640 supplemented with 2 mM L-glutamine, 200 IU/mL penicillin/100 µg/mL streptomycin and 10% fetal bovine serum; all from Life Technologies, Basel, Switzerland). Cells were pre-treated with Ze339, petasin, neopetasin, isopetasin, FAT or zileuton (0–40 µg/mL) for 15 min at 37 °C in a humidified atmosphere with 5% CO₂. Eosinophils were primed with GM-CSF (25 ng/mL) for 20 min and then stimulated with either PAF (100 nM) or C5a (10 nM) for 25 min at 37 °C. Non-specific stimulations were performed using unprimed eosinophils ($1 \times 10^5/\text{mL}$) and A23187 (1 µM). After stimulations, supernatants were harvested and stored at –20 °C until analysis (cysteinyll-Ts and ECP).

Immunoassays

Cysteinyll-Ts were measured using the CAST-ELISA from Böhmann Laboratories (Allschwil, Switzerland). ECP levels were measured using the UniCAPTM System for ECP (Pharmacia & Upjohn, Dubendorf, Switzerland) according to the manufacturer's instructions.

Intracellular calcium measurements

Changes in $[\text{Ca}^{2+}]_i$ were assayed in human eosinophils as previously described [31,32]. Briefly, cells were resuspended at $5\text{--}10 \times 10^6/\text{mL}$ in complete culture medium and incubated with 10 µL of a 1-mM stock solution of the

acetoxymethylester derivative of Fura-2 for 20 min at 37 °C. Extracellular dye was then removed by washing and cells were resuspended at $2 \times 10^6/\text{mL}$ in complete culture medium and stored in the dark until analysis at 37 °C. Cells were washed and resuspended ($1 \times 10^6/\text{mL}$) in buffer A (in mM: NaCl 140, KCl 3, MgCl₂ 1, glucose 10, CaCl₂ 1 and HEPES 20, pH 7.23) immediately before use. Cells were continuously monitored and stirred in a heated (37 °C) quartz cuvette in a fluorospectrometer ($\lambda_{\text{excitation}} = 340/380$ nm, $\lambda_{\text{emission}} = 505$ nm) and analysed with the DM3000 Cation Measurement software (Spex Industries Inc., Edison, NJ, USA). Each run was calibrated by addition of 1 µM ionomycin and 0.2% Triton X-100 followed by 10 mM EGTA. Changes in cytosolic free calcium ($\Delta[\text{Ca}^{2+}]_i$) were calculated as the peak value obtained within the first minute following PAF (100 nM) or C5a (10 nM) stimulation.

Enzymatic activity of cPLA₂

cPLA₂ activity in intact eosinophils (10^6 cells/mL) was assessed by measuring the release of [³H]-AA/metabolites from labelled cells [33]. Radiolabeling of cells with [³H]-AA was achieved by including 0.5 µCi/mL [³H]-AA in RPMI-1640 supplemented with 0.2% BSA for 120 min. The [³H]-AA that had not been incorporated into cellular lipids was removed by washing the cells three times with RPMI-1640/0.2% BSA. Cells were resuspended in RPMI-1640/0.2% BSA ($1 \times 10^6/\text{mL}$) and pre-incubated with TFMK, zileuton, petasin, isopetasin or isopetasin for 20 min at 37 °C and then stimulated with 1 µM A23187 for 25 min. The supernatants were harvested and assayed for radioactivity by liquid scintillation counting (Kontron Instruments, Zurich, Switzerland).

5-LO expression analysis

Expression of 5-LO was assessed by immunofluorescence analysis. Briefly, freshly isolated eosinophils or neutrophils (10^5 cells/mL) were stimulated with 1 µM A23187 in 1% BSA-PBS for 25 min at 37 °C, followed by fixating and permeabilizing cells with a formaldehyde/saponin solution (Ortho Permeafix[®]). After washing with 1% BSA-PBS, cells were incubated with 10 µg/mL primary anti-5-LO or control mAb for 20 min, washed again, and incubated with 0.5 µg/mL secondary FITC-labelled mouse IgG₁ antibody for another 20 min. Flow cytometric analysis was performed using an EPICS XL (Coulter, Hialeah, FL, USA).

5-LO translocation assay

Translocation of 5-LO was performed using indirect immunofluorescence microscopy. Briefly, eosinophils

(5×10^4 cells/mL), resuspended in 1% BSA-PBS, were pre-incubated with vehicle or drug, and stimulated with $1 \mu\text{M}$ A23187 for 25 min at 37°C , followed by cytocentrifugation (Shandon Southern Products, Runcorn, UK) on superfrost plus glass slides at 7.06 g for 1 min [15,34]. Cells were fixed in -20°C methanol for 30 min, permeabilized with -20°C acetone for 3 min and blocked with 1% BSA-PBS containing 20% non-immune normal rabbit serum (NRS) for 45 min. Primary anti-5-LO mAb ($10 \mu\text{g/mL}$) was prepared in 1% BSA-PBS containing 5% NRS and applied for 30 min. After washing of the mounts, cytopins were incubated with CyTM3-conjugated donkey anti-mouse Ab diluted 1 : 200 in 1% BSA-PBS containing 5% NRS for 30 min. All washes were conducted as follows: two times PBS, two times PBS/0.2% Tween, and again two times PBS. Control slides were routinely performed in the presence of an irrelevant mAb and showed no fluorescent development. Fluorescence was visualized by using an Axiovert 405 M microscope from Zeiss (Oberkochen, Germany) at a magnification of $\times 400$.

Statistical analysis

Inhibition of induced LT synthesis, ECP release and $[\text{Ca}^{2+}]_i$ -changes by the compounds were calculated in percentage related to control cells (pre-incubated without compound). Data are presented as means $\pm$ SEM. The Mann-Whitney rank sum test was used to determine statistical differences ($*P < 0.05$).

Results

A mixture of petasins inhibits synthesis of cysteinyl-LT

To determine the active compounds of Ze339, a preliminary screening was performed using the two main fractions PIN and FAT. The decision to focus on these two fractions was based on the previously described inhibition of LT synthesis by petasin and isopetasin [27,28]. Eosinophils ($1 \times 10^5/\text{mL}$) were incubated in complete culture medium with Ze339, PIN or FAT before stimulation with calcium ionophore A23187 ($1 \mu\text{M}$). As shown in Fig. 2, Ze339 showed strong inhibitory activity with an IC_{50} of approximately $16 \mu\text{g/mL}$ ($54.0 \pm 8.2\%$) and a maximal inhibition at $40 \mu\text{g/mL}$ ($92.2 \pm 0.4\%$). PIN was less active and maximal inhibition of cysteinyl-LT generation was observed at $40 \mu\text{g/mL}$ ($69.9 \pm 1.1\%$). Higher concentrations of the drugs did not further inhibit the generation of LTs (not presented). The residual FAT fraction of Ze339 had only little effect on cysteinyl-LT synthesis (inhibition at $40 \mu\text{g/mL}$ was $26.3 \pm 7.0\%$). Since the pharmacologically active substances of PIN appear to be the stereoisomers petasin, isopetasin and neopetasin, the following

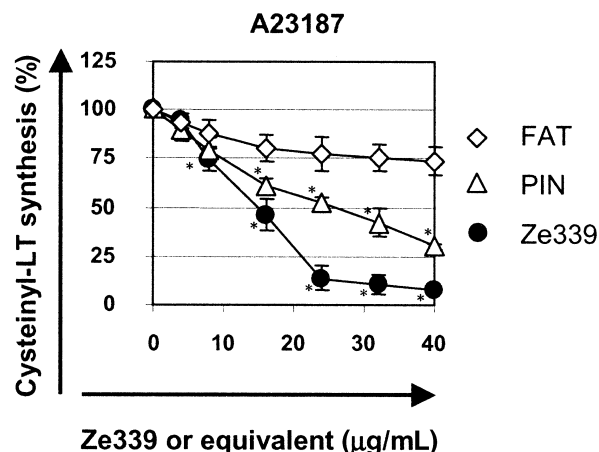


Fig. 2. Inhibition of A23187-induced cysteinyl-LT synthesis by Ze339 and its two separated fractions PIN and FAT in eosinophils. PIN and FAT were used in concentrations corresponding to their content in Ze339 (29.2% for PIN and 60.9% for FAT, respectively). Maximal cysteinyl-LT synthesis among independent experiments ranged between 3500 and 6500 pg/mL. Inhibition of cysteinyl-LT synthesis is expressed as percentage related to control cells stimulated without inhibitor. Values are means $\pm$ SEM of four independent experiments ($*P < 0.05$).

experiments were performed using these three defined compounds.

Petasin, isopetasin and neopetasin inhibit cysteinyl-LT synthesis

Purified eosinophils were incubated with increasing concentrations of petasin, isopetasin and neopetasin. Zileuton, a commercially available 5-LO inhibitor, was used as positive control in the same concentrations. Eosinophils ($1 \times 10^6/\text{mL}$) were then primed with GM-CSF and stimulated with either C5a or PAF (no significant LT synthesis was observed using unprimed eosinophils, not presented). As shown in Fig. 3, all compounds inhibited C5a- and PAF-mediated cysteinyl-LT synthesis in GM-CSF-primed eosinophils in a dose-dependent manner. The source of eosinophils (control individuals or allergic patients) did not matter in these assays. Inhibitor concentrations above $40 \mu\text{g/mL}$ did not further inhibit the generation of LTs (not presented). No significant differences were observed among the blocking compounds (IC_{50} was $< 24 \mu\text{g/mL}$ in each case). At a concentration of $40 \mu\text{g/mL}$, the cysteinyl-LT synthesis inhibition ranged between 68% and 82% in both C5a- and PAF-stimulated cells. In addition, petasin, isopetasin and neopetasin and zileuton showed the same inhibitory effects on A23187-stimulated cysteinyl-LT synthesis in eosinophils (not presented). Moreover, montelukast, a commercially available

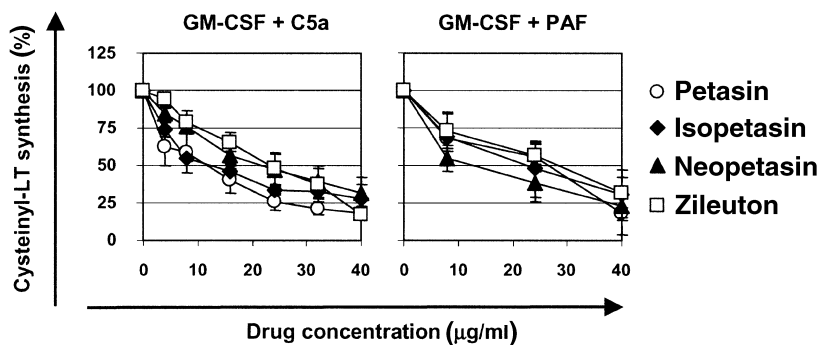


Fig. 3. Petasin, isopetasin and neopetasin inhibit C5a- and PAF-induced cysteinyl-LT synthesis in a dose-dependent manner in GM-CSF-primed eosinophils. As a control, the 5-LO inhibitor zileuton was used. No significant differences between the compounds were found. Maximal cysteinyl-LT synthesis among independent experiments ranged between 400 and 4000 pg/mL. Inhibition of cysteinyl-LT synthesis is expressed as percentage related to control cells stimulated without inhibitor. Values are means $\pm$ SEM of four to nine independent experiments (* $P < 0.05$).

LT-receptor antagonist, had no inhibitory activities on LT generation in the same concentration range. Also, it is unlikely that the inhibitory effect of the three different petasins on cysteinyl-LT synthesis was the consequence of apoptosis induction, since concentrations of up to 80 μ g/mL had no effect on eosinophil apoptosis in 24-h cultures (not presented).

Petasin, but not isopetasin or neopetasin, inhibits ECP release

Both C5a and PAF stimulated ECP release in GM-CSF-primed eosinophils. In contrast, no significant ECP release was observed in unprimed cells (not presented). As shown in Fig. 4, petasin, but not isopetasin, neopetasin or zileuton, significantly blocked C5a- and PAF-stimulated ECP release in GM-CSF-primed eosinophils. At 40 μ g/mL, petasin-mediated inhibition of ECP release was $89.1 \pm 1.9\%$ in PAF-stimulated and $80.7 \pm 8.3\%$ in C5a-stimulated cells. The IC_{50} for petasin was $< 8 \mu$ g/mL (at 8 μ g/mL we observed $63.0 \pm 7.9\%$ inhibition in PAF-stimulated and $52.2 \pm 8.0\%$ in C5a-stimulated cells). Isopetasin, neopetasin and zileuton had no significant inhibitory activity in this model of eosinophil activation.

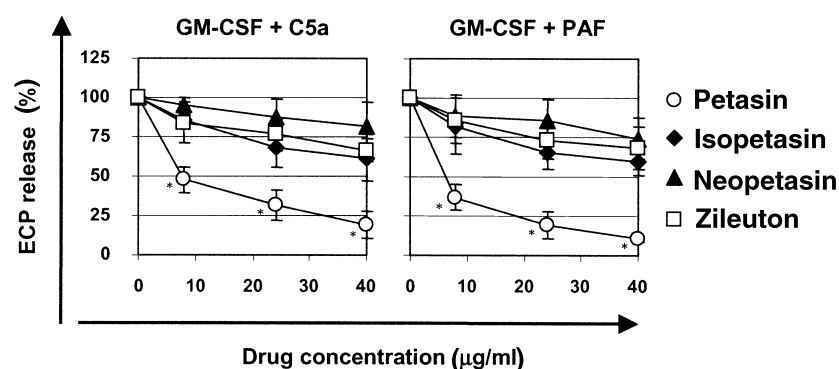


Fig. 4. Petasin, but not isopetasin or neopetasin, inhibits C5a- and PAF-induced ECP release in a dose-dependent manner in GM-CSF-primed eosinophils. The 5-LO inhibitor zileuton had no inhibitory effect. Maximal ECP release among independent experiments ranged between 86 and 207 μ g/mL. Inhibition of ECP release is expressed as percentage related to control cells stimulated without any inhibitor. Values are means $\pm$ SEM of four independent experiments (* $P < 0.05$).

Petasin, but not isopetasin or neopetasin, suppresses cPLA₂ activity

As shown in Fig. 5a, petasin inhibited A23187-stimulated cPLA₂ activity in eosinophils with the same efficacy as optimal concentrations of the specific cPLA₂ inhibitor TFMK. In contrast, isopetasin, neopetasin and zileuton had no inhibitory effect. In parallel experiments, the synthesis of cysteinyl-LT was measured. In spite of different effects on cPLA₂ activity, all compounds showed a drastic inhibition of cysteinyl-LT synthesis (Fig. 5b). Investigations with higher drug concentrations (up to 40 μ g/mL) revealed the same effects on cPLA₂ activity and cysteinyl-LT synthesis (not presented). These data suggest that the different inhibitory capacity of petasin and its stereoisomers on ECP release might be due to their different ability to block cPLA₂ activity.

Petasin, but not isopetasin or neopetasin, suppresses 5-LO translocation from the cytosol to the nuclear envelope

To determine the immunoreactivity of the anti-5-LO mAb, we first determined intracellular 5-LO protein levels by flow cytometry. As shown in Fig. 6a, the presence of 5-LO in purified eosinophils was clearly detectable and 5-LO

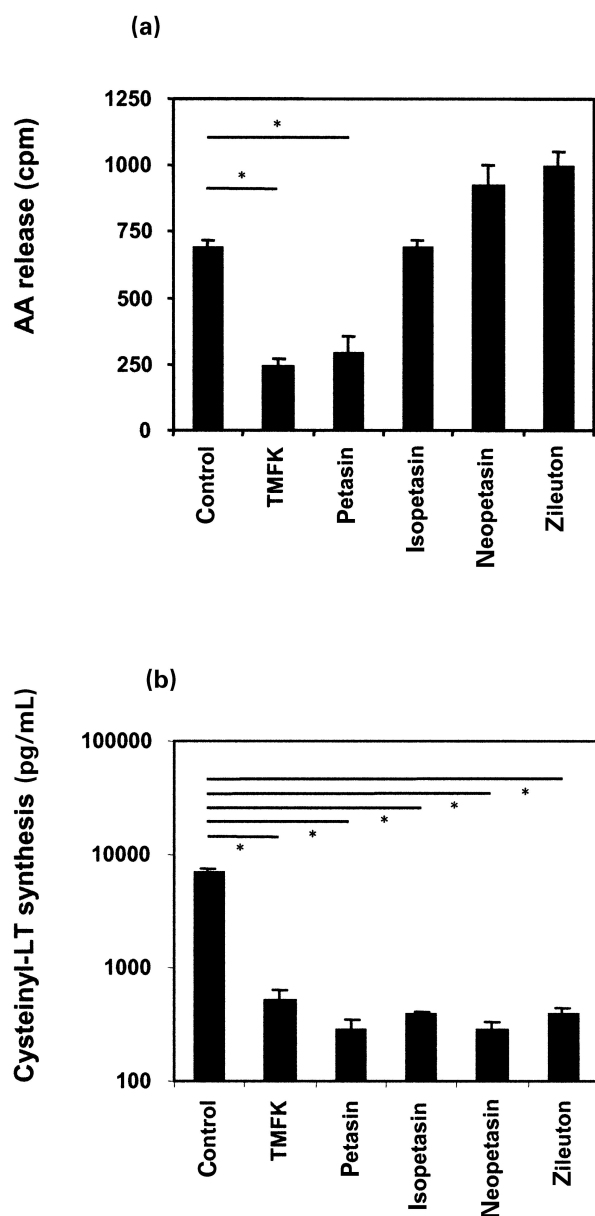


Fig. 5. Petasin, but not isopetasin or neopetasin, inhibits A23187-induced cPLA₂ enzymatic activity in eosinophils. (a) [³H]AA release was significantly inhibited by petasin (24 µg/mL) and TFMK (1 µM, positive control). In contrast, we observed no significant reduction of released [³H]AA when cells were pre-incubated with isopetasin, neopetasin or zileuton (all at 24 µg/mL). The same results were observed using petasin concentrations of 40 µg/mL. (b) In spite of differences in inhibiting the enzymatic activity of cPLA₂, all compounds block cysteinyl-LT synthesis. Values are means ± SEM of four independent experiments (**P* < 0.05).

expression appeared to be higher in eosinophils compared with neutrophils. We next investigated 5-LO localization by indirect immunofluorescence microscopy (Fig. 6b). In resting cells, 5-LO was located in the whole nucleus and in the cytoplasm around the nucleus envelope. In contrast, A23187-stimulated eosinophils demonstrated a different pattern: 5-LO appeared to be mostly located at the nuclear membrane. Whereas petasin prevented the A23187-induced translocation of 5-LO, isopetasin, neopetasin and zileuton did not. These data suggest that isopetasin, neopetasin or zileuton block LT synthesis at the level of or distal to 5-LO.

Petasin, but not isopetasin or neopetasin, suppresses PAF- and C5a-mediated increases in [Ca²⁺]_i

PAF-induced [Ca²⁺]_i changes were monitored over 250 s. Cells were from different donors for each drug tracing and therefore maximal [Ca²⁺]_i changes ranged between 289 and 360 nM. As shown in Fig. 6 and Table 1, pretreatment with 16 µg/mL petasin completely blocked PAF-induced increases in [Ca²⁺]_i in eosinophils. In contrast, isopetasin, neopetasin and zileuton pre-incubation reduced only partially PAF-induced intracellular calcium mobilization. Similar results were obtained in C5a-stimulated eosinophils (Table 1). These results suggest that petasin blocks PAF- and C5a-induced signal transduction pathways at the level of or proximal to PLC_β. In contrast, the stereoisomers isopetasin and neopetasin do not have this capacity.

Discussion

Eosinophils are important inflammatory effector cells that contribute to tissue damage due to the release of toxic mediators in several allergic diseases. Besides the generation of toxic molecules, eosinophils appear to amplify inflammatory responses by generating cytokines, chemokines and lipid mediators. Blocking of both degranulation of cationic proteins and generation of inflammatory mediators seems to be one reasonable strategy to improve the clinical situation of patients where activated eosinophils are part of the pathogenic process. Indeed, the inhibition of actions of eosinophil-derived LT has been shown to be beneficial for patients with bronchial asthma. There are two strategies to block LTs: (a) by synthesis blockers, such as 5-LO [35] or FLAP [36] inhibitors, or (b) by LT receptor antagonists [37].

In previous chemical studies, petasin, isopetasin and neopetasin have been shown to be stereoisomers with a chemical structure belonging to the sesquiterpene esters [25]. In this study, we investigated the effect of the three petasin stereoisomers in two *in vitro* models, which reflect eosinophil functions. With the generation of LT, we analysed the potential of the drugs to block the synthesis

of at least one class of inflammatory mediators. The capacity of petasins to block eosinophil degranulation was measured by the release of ECP. In both models, priming of eosinophils was done by short-term incubation with GM-CSF. It has previously been shown that IL-3, IL-5 and GM-CSF strongly enhance the response of eosinophils to different agonists [5–8]. After optimal stimulation with C5a or PAF, primed eosinophils generated large amounts of

cysteinyl-LT and released significant amounts of ECP into the supernatant. In both assays, priming with GM-CSF was absolutely essential since both C5a and PAF alone neither significantly increased LT synthesis nor ECP release.

Petasin, isopetasin and neopetasin inhibited cysteinyl-LT synthesis in both C5a- and PAF-stimulated primed eosinophils. The pharmacological activity of the petasins appeared to be in the same range as observed using the known 5-LO blocker zileuton [35]. In contrast, ECP release was blocked by petasin, but not by the epimers isopetasin and neopetasin, in the same two systems of eosinophil stimulation. Zileuton had, as expected, no effect on ECP release. The different activities among the different petasins were somewhat surprising. Therefore, we analysed some key intracellular signalling events following eosinophil stimulation in order to understand the different modes of action of the three investigated petasins.

Although all used petasin stereoisomers blocked C5a- and PAF-induced cysteinyl-LT production, only petasin inhibited A23187-induced cPLA₂ activity and 5-LO translocation to the nuclear envelope. This implies that the molecular target of petasin is at the level of or proximal to cPLA₂ activation. Our experiments do not exclude the possibility that petasin blocks the mitogen-activated protein kinase (MAPK) pathway. Isopetasin and neopetasin had no effect in these two systems, suggesting that they either block the enzymatic activity of 5-LO (as zileuton does) or distal events within the cysteinyl-LT synthesis pathway (see Fig. 8). These particular experiments did not exclude the possibility that isopetasin and neopetasin target molecules prior to calcium upregulation or components of the MAPK pathway, since these signal transduction pathways are bypassed by A23187. However, since isopetasin and neopetasin do not significantly block C5a- and PAF-induced increases in $[Ca^{2+}]_i$ and ECP release, it is unlikely that these two compounds would indeed act proximal to activation of cPLA₂.

Further evidence for potential differences in targeting intracellular molecules between the three different petasins used in this study were obtained by intracellular calcium measurements in stimulated eosinophils. Petasin, but not isopetasin, neopetasin or zileuton, significantly prevented both PAF- and C5a-induced increases in $[Ca^{2+}]_i$ in eosinophils. Thus, using optimal concentrations, petasin may reduce PAF- and C5a-mediated increases in $[Ca^{2+}]_i$, below the levels needed for cPLA₂ activation and 5-LO translocation (Fig. 8). However, the question remains why 16 µg/mL petasin completely blocks PAF- and C5a-induced calcium transients, but only partially inhibits cysteinyl-LT synthesis. Does this mean that a calcium-independent cPLA₂ exists? Because petasin blocked signalling events at the level of or proximal to cPLA₂ activation, this study did not answer the question of

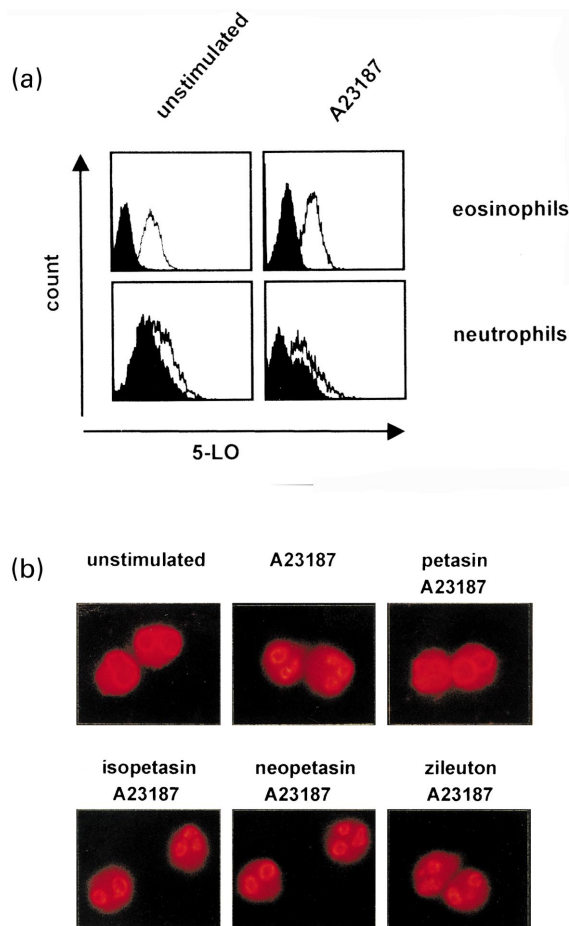


Fig. 6. Petasin, but not isopetasin or neopetasin, inhibits A23187-induced 5-LO translocation to the nuclear membrane in eosinophils. (a) Expression of 5-LO in eosinophils and neutrophils as assessed by flow cytometry. Eosinophils appeared to have a higher intracellular 5-LO content compared with neutrophils. (b) Detection of 5-LO translocation by indirect immunofluorescence microscopy in eosinophils. Except petasin, all other compounds do not prevent 5-LO accumulation around the nucleus following A23187 stimulation. Eosinophils stained with control antibody showed no fluorescence (not presented). The data are representative of at least three independent experiments.

Table 1. C5a- and PAF-mediated increases in $[Ca^{2+}]_i$ are significantly inhibited by petasin, but not isopetasin, neopetasin, or zileuton

Concentration	Petasin	Isopetasin	Neopetasin	Zileuton
<i>C5a</i>				
0	100	100	100	100
4 $\mu\text{g/mL}$	59.6 $\pm$ 10.5*	88.0 $\pm$ 7.1	73.0 $\pm$ 9.6	87.0 $\pm$ 3.0
8 $\mu\text{g/mL}$	24.9 $\pm$ 6.1*	62.3 $\pm$ 0.8	69.0 $\pm$ 8.7	80.5 $\pm$ 1.9
16 $\mu\text{g/mL}$	0.7 $\pm$ 0.7*	43.3 $\pm$ 5.2	63.5 $\pm$ 9.9	62.6 $\pm$ 5.1
<i>PAF</i>				
0	100	100	100	100
4 $\mu\text{g/mL}$	73.0 $\pm$ 1.3	74.8 $\pm$ 11.4	92.2 $\pm$ 3.7	93.7 $\pm$ 2.6
8 $\mu\text{g/mL}$	58.3 $\pm$ 1.5*	67.5 $\pm$ 10.0	79.6 $\pm$ 5.2	78.3 $\pm$ 9.8
16 $\mu\text{g/mL}$	1.1 $\pm$ 1.1*	60.7 $\pm$ 8.3	57.8 $\pm$ 6.4	70.8 $\pm$ 8.3

Experiments were performed as described in 'Materials and methods' and run as shown in Fig. 7. The values represent percentages of the original peak calcium levels and are means $\pm$ SEM of four independent experiments. At 16 $\mu\text{g/mL}$, petasin, but not isopetasin, neopetasin or zileuton, completely blocked ligand-induced calcium mobilization (* $P < 0.05$).

whether petasin is able to inhibit the more distal LT pathway as isopetasin and neopetasin do.

Previously published work suggested that cPLA₂ is not only activated by increases in $[Ca^{2+}]_i$, but also by serine/threonine kinases of the MAPK pathway [38,39]. Recently, some progress has been made in respect of our understanding of how eosinophil priming may work at the molecular level. Stimulation of the common β -subunit of the IL-3/IL-5/GM-CSF receptor results in activation of this pathway [40], but it may not reach the activation level needed for functional responses [41]. Increased activation of the MAPK pathway might be achieved due to concurrent activation of G-protein-coupled receptors via PKC. PKC may either stimulate Ras^{GTP} by inhibiting Ras GTPase activating protein [42] or Raf via activation of sphingosine kinase and sphingosine 1-phosphatase [43]. Although these

PKC actions have been shown in other cell systems, it is clear that the MAPK pathway can also be activated via G-protein-coupled receptors in eosinophils, resulting in both increased LT production [41] and degranulation of cationic proteins [44]. In such a scenario, a drug that acts at the level of or proximal to PLC β (e.g. petasin) would block cPLA₂ activity in two ways: (a) by not providing sufficient amounts of cytosolic free calcium, and (b) by inadequate activation of the enzyme via the MAPK pathway (see Fig. 8).

Many of our current anti-allergic and/or anti-asthmatic groups of drugs, such as anti-cholinergic drugs, chromones and phosphodiesterase inhibitors, have herbal origins [23]. Thus, in spite of many reservations about alternative medicine among physicians today, it is recommended to distinguish between different approaches. The identification

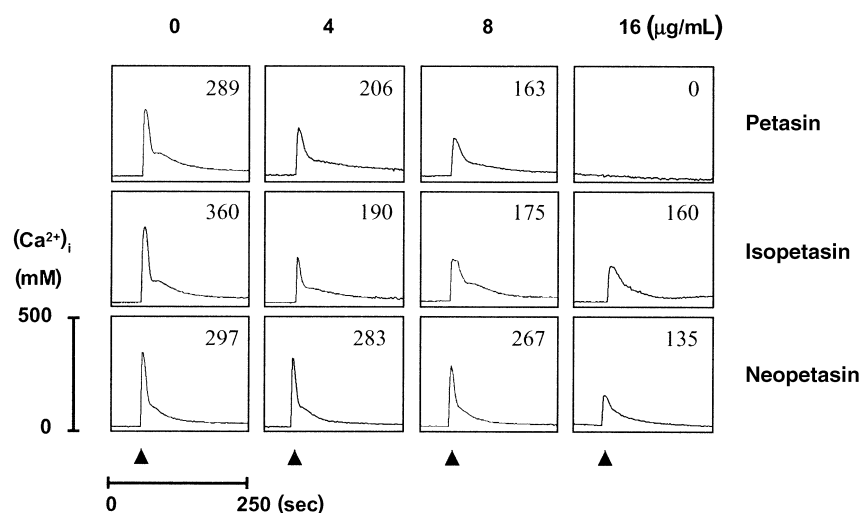


Fig. 7. Petasin, but not isopetasin or neopetasin, inhibits PAF-induced increases in $[Ca^{2+}]_i$ in eosinophils. At 16 $\mu\text{g/mL}$, petasin completely abrogated the calcium signal. PAF was added where indicated ($\blacktriangle$). The tracings are representative of four independent experiments.

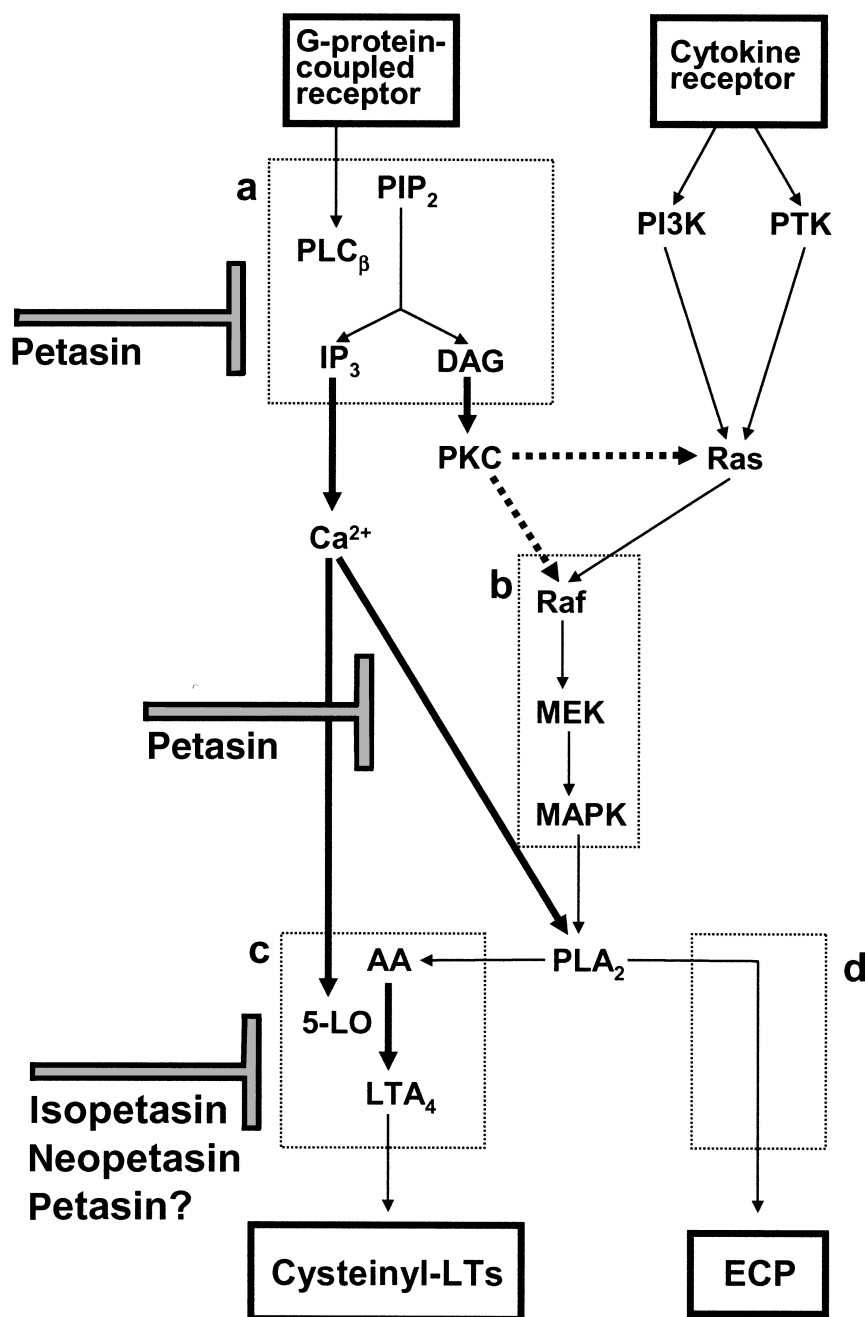


Fig. 8. A simplified scheme suggesting how priming signals (e.g. IL-3, IL-5, GM-CSF) and activation signals (e.g. C5a, PAF) are transduced in eosinophils. Upon stimulation, G-protein-coupled receptors immediately activate the inositol polyphosphate cascade (a), resulting in increases in $[Ca^{2+}]_i$ and activation of protein kinase C (PKC). Via PKC, G-protein-coupled receptors are linked to the MAPK pathway (b), although the exact mechanism is unknown in eosinophils. Activation of the common β -subunit of the IL-3/IL-5/GM-CSF receptor is also associated with MAPK cascade activation. Only concurrent, but not single, stimulation of G-protein-coupled and cytokine receptors may reach the threshold needed to phosphorylate cPLA₂. cPLA₂ activation, which also requires calcium, is essential for both generation of LTs (c) and degranulation (d). Results of this study suggest that petasin blocks the inositol polyphosphate cascade and A23187-induced cPLA₂ activity that may result in both inhibition of LT synthesis and ECP release. However, additional actions within the MAPK and LT pathways cannot be excluded. Isopetasin and neopetasin specifically block the LT pathway, whereas degranulation is not influenced.

of active compounds and their functional effects in established *in vitro* and *in vivo* models is a rather scientific approach and can therefore be integrated into orthodox practice. We believe that we have made progress in our understanding of how components of the *petasites hybridus* extract act within inflammatory cells and some functional consequences.

The exact molecular targets of petasin as well as isopetasin and neopetasin remain to be established. Moreover, it is unclear whether these compounds may

have similar effects in other inflammatory effector cells in allergic reactions, such as mast cells. Clearly, the reduction of mast cell-derived LT production and histamine release by petasin would suggest an even higher anti-allergic potential of these compounds. With the discovery that different petasins block different cell functions, it might be possible to develop two classes of drugs: (a) drugs that specifically block the LT pathway, and (b) drugs that block inflammatory responses in a rather non-specific manner. Clinical studies with petasins

are underway to investigate their clinical relevance as anti-allergic drugs *in vivo*.

Acknowledgements

This work was supported by grants from Max Zeller Söhne AG, Herbal Remedies, Romanshorn, Switzerland, the Swiss National Science Foundation (Grant no. 31-58916.99) and EMDO Foundation, Zurich. We thank Dr T. Hartung (University of Konstanz, Konstanz, Germany) for GM-CSF; Dr B. Truempi (Abbott AG, Baar, Switzerland) for the generous gift of zileuton; Dr L. Borer (Merck Sharp Dohme-Chibret, Glatbrugg, Switzerland) for the generous gift of montelukast; Dr A. Trautmann for advice concerning immunofluorescence stainings; M. Weber and B. Rückert for excellent technical assistance; and Prof. A. Brattström and Dr B. Debrunner for continuous support and critical discussions.

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