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Biochemical Pharmacology 61 (2001) 1041–1047

Biochemical Pharmacology

Role of petasin in the potential anti-inflammatory activity of a plant extract of *petasites hybridus*

Olivier A.R. Thomet[a,b,c], Ulrich N. Wiesmann[b], Andreas Schapowal[d], Christian Bizer[e], Hans-Uwe Simon[a,c,*]

[a]*Swiss Institute of Allergy and Asthma Research (SIAF), Davos, Switzerland*
[b]*Department of Metabolic Diseases, Children's Hospital, University of Bern, Bern, Switzerland*
[c]*Department of Pharmacology, University of Bern, Friedbühlstrasse 49, CH-3010 Bern, Switzerland*
[d]*ORL/Allergology Clinical Office, Landquart, Switzerland*
[e]*High-Altitude Clinic Davos-Wolfgang, Davos, Switzerland*

Received 2 September 2000; accepted 23 November 2000

Abstract

A large production of leukotrienes (LTs) can be induced in human eosinophils or neutrophils by priming with granulocyte-macrophage colony-stimulating factor and subsequent stimulation with platelet-activating factor (PAF) or the anaphylatoxin C5a. Here, we investigated the effects of a plant extract of *petasites hybridus* (Ze339) and its isolated active sesquiterpene ester petasin in these two *in vitro* cell models. Zileuton, a 5-lipoxygenase inhibitor, was used as a positive control. All compounds inhibited both cysteinyl-LT synthesis in eosinophils and LTB_4 synthesis in neutrophils. In contrast, only Ze339 and petasin, but not zileuton, abrogated PAF- and C5a-induced increases in intracellular calcium concentrations. These data suggest that Ze339 and petasin may block, compared to zileuton, earlier signalling events initiated by G protein-coupled receptors in granulocytes, perhaps at the level of or proximal to phospholipase C_β. Taken together, petasin appears to be one major active compound of *petasites hybridus* extract, since it demonstrates the same inhibitory activities on calcium fluxes and subsequent LT generation in both eosinophils and neutrophils as Ze339 does.

Keywords: Eosinophils; Leukotrienes; Neutrophils; Petasin; *Petasites hybridus;* Signal transduction

1. Introduction

Increased numbers of neutrophils are observed in inflammatory responses caused by bacteria [1,2]. In contrast, eosinophils are important effector cells, which accumulate at sites of parasitic and other non-bacterial inflammations [3,4]. However, the presence of either eosinophils or neutrophils alone does not cause an inflammatory response. To develop inflammatory symptoms, granulocytes need to be activated [5,6]. There have been a number of *in vitro* studies describing eosinophil and neutrophil activation mechanisms. Functional responses of eosinophils and neutrophils to various agonists, including lipid mediators, complement factors, or chemokines [7–9], are increased by hematopoietins such as IL-3 and GM-CSF. This effect of hematopoietins, called "priming", is also observed in basophils and macrophages [10,11]. For both LT synthesis [8,12] and degranulation [13,14], priming of granulocytes appears to be required.

Lipid mediators (e.g. PAF) and complement factors (e.g. C5a) bind to G protein-coupled receptors and activate phospholipase C_β [15], which catalyses hydrolysis of phosphatidylinositol-4,5-biphosphate to IP_3 and diacylglycerol. IP_3-mediated increases in $[Ca^{2+}]_i$ are essential for the translocation of $cPLA_2$ from the cytosol to the nuclear membrane [16]. At the nuclear membrane, $cPLA_2$ liberates AA from phospholipids [17]. Free AA is transferred to FLAP, which is also located in the nuclear envelope to generate LTs. Translocation of 5-LO from cytosol to FLAP on membrane compartments also appears to be $[Ca^{2+}]_i$-

* Corresponding author. Tel.: +41-31-632-3281; fax: +41-31-632-4992.

E-mail address: hus@pki.unibe.ch (H.-U. Simon).

Abbreviations: AA, arachidonic acid; $[Ca^{2+}]_i$, cytosolic free calcium concentration; $cPLA_2$, cytosolic phospholipase A_2; C5a, complement factor C5a; FLAP, 5-LO-activating protein; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; IP_3, inositol trisphosphate; 5-LO, 5-lipoxygenase; LT, leukotriene; MAPK, mitogen-activated protein kinase; mAb, monoclonal antibody; PAF, platelet-activating factor; and Ze339, CO_2 estract of *petasites hybridus*.

0006-2952/01/$ – see front matter
PII: S0006-2952(01)00552-4

dependent and essential for the generation of LTA_4 from AA as shown by immunoblot analysis [18] and immunofluorescence [19].

In human neutrophils, monocytes, and macrophages, LTA_4 is predominantly converted into LTB_4 by LTA_4 hydrolase [20,21]. LTB_4 stimulates superoxide generation and lysosomal enzyme release, and amplifies leukocyte chemotaxis as well as adhesion in bacterial-infected tissues [22–24]. On the other hand, LTA_4 is conjugated with reduced gluthatione by LTC_4 synthase to form LTC_4 [25–27] in both human eosinophils and macrophages. 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 [28]. To prevent LT actions, new synthetic drugs such as 5-LO inhibitors (e.g. zileuton), LTB_4 receptor antagonists (e.g. LY-223982), cysteinyl-LT receptor antagonists (e.g. montelukast), and FLAP inhibitors (e.g. MK-0591) have been developed [29,30].

The plant extract of *petasites hybridus* (Ze339) contains the sesquiterpene ester petasin as its main active compound. Inhibition of cysteinyl-LT synthesis by extracts of *petasites hybridus* and petasin have been observed in rat peritoneal macrophages [31] and whole leukocyte populations [32]. In this article, we compared the effects of Ze339 and petasin to those of zileuton on the generation of cysteinyl-LTs in eosinophils and LTB_4 in neutrophils as well as their pharmacological activities on intracellular calcium mobilisation, a key mechanism in the activation of $cPLA_2$ and 5-LO.

2. Materials and methods

2.1. Reagents

Ficoll separating solution was from Biochrom KG. GM-CSF was a gift from Dr. T. Hartung. PAF was obtained from Calbiochem. Complement factor C5a and calcium ionophore A23187 were purchased from Sigma Chemical Co. Fura-2-acetoxymethylester and ionomycin were from Roche Diagnostics. Zileuton was a gift from Abbott. The high-pressure CO_2 extract of *petasites hybridus* (Ze339) and its isolated active compound petasin [33] were provided by Max Zeller Söhne AG. Anti-CD16 mAb microbeads were purchased from Milteny Biotec. Unless stated otherwise, all other reagents were from Sigma.

2.2. Composition of Ze339

After standardisation, Ze339 contained 14.1% of the active compound petasin in addition to 85.1% residual compounds, mainly represented by inactive apolar fatty acids as well as inactive polar compounds. Petasin was isolated from Ze339 to a purity of at least 99% [33].

2.3. Purification of eosinophils, neutrophils, and macrophages

Peripheral blood eosinophils were isolated by negative selection from patients with atopic dermatitis or asthma [34–36]. Neutrophils were purified from healthy donors [34], and macrophages were collected from bronchoalveolar lavage fluids of asthmatic patients [35]. Informed consent was obtained from all patients and healthy donors. The study was approved by the Swiss Academy of Medical Science (Medical Ethics Committee of Davos).

2.4. Stimulation of eosinophils, neutrophils, and macrophages

Unless stated otherwise, either 1×10^6/mL of purified eosinophils, 1×10^6/mL of macrophages, or 1×10^7/mL of neutrophils were cultured in complete culture medium (RPMI-1640 supplemented with 2 mM L-glutamine, 200 IU/mL of penicillin/100 μg/mL of streptomycin, and 10% foetal bovine serum; all from Life Technologies). Cells were pretreated with Ze339, petasin, or zileuton (0–40 μg/mL) for 15 min at 37° in a humidified atmosphere with 5% CO_2. Cells were primed with GM-CSF (50 ng/mL) for 20 min and then stimulated with PAF (100 nM) or C5a (10 nM) for 25 min at 37°. After stimulation, supernatants were harvested and stored at −20° until analysis for the presence of cysteinyl-LTs or LTB_4.

2.5. Immunoassays

Cysteinyl-LTs were measured using the cellular allergen stimulation test (CAST)-ELISA from Bühlmann Laboratories. LTB_4 was measured using the LTB_4-ELISA from R&D Systems.

2.6. Intracellular calcium measurements

Changes in $[Ca^{2+}]_i$ were assayed in human eosinophils and neutrophils as previously described [37,38]. Briefly, cells were resuspended at 5 to 10×10^6/mL in complete culture medium and incubated with 10 μL of a 1-mM stock solution of fura-2-acetoxymethylester for 20 min at 37°. Extracellular dye was then removed by washing, and cells were resuspended at 2×10^6/mL in complete culture medium and stored in the dark until analysis at 37°. Cells were washed and resuspended (1×10^6/mL) in buffer A (in mM: NaCl 140, KCl 3, $MgCl_2$ 1, glucose 10, $CaCl_2$ 1, and HEPES 20, pH 7.23) immediately before use. Cells were continuously monitored and stirred in a 1.8-mL heated (37°) quartz cuvette in a fluorospectrometer ($\lambda_{excitation}$ = 340/380 nm, $\lambda_{emission}$ = 505 nm) and analysed with the DM3000 Cation Measurement software (Spex). 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 ($\Delta[Ca^{2+}]_i$) were calculated as the peak value

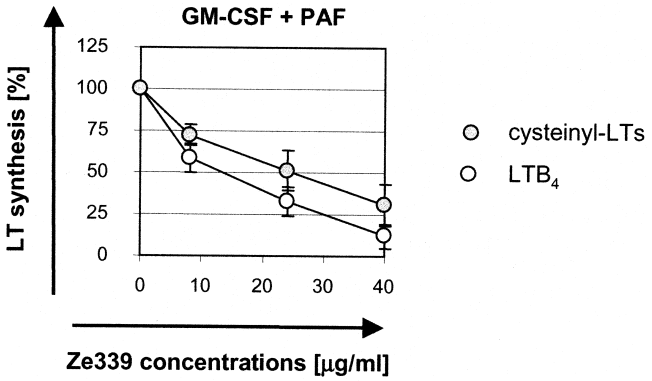

Fig. 1. Inhibition of PAF-induced cysteinyl-LT and LTB_4 synthesis by Ze339 in macrophages. Maximal synthesis in independent experiments ranged between 300–600 pg/mL for cysteinyl-LTs and 10,000–28,500 pg/mL for LTB_4. Inhibition of LT synthesis is expressed as percent related to control cells stimulated without inhibitor. Values are means ± SEM of four independent experiments ($*P < 0.05$).

obtained within the first minute following PAF (100 nM) or C5a (10 nM) stimulation.

2.7. Statistical analysis

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

3. Results

3.1. Inhibition of cysteinyl-LT and LTB_4 synthesis by Ze339 in macrophages

To determine the inhibitory activity of Ze339 on the synthesis of cysteinyl-LTs and LTB_4, a preliminary screening was performed using lung macrophages based on the previously described cysteinyl-LT synthesis [31]. Macrophages from bronchoalveolar lavage fluids (1×10^6/mL) were incubated in complete culture medium with Ze339 before stimulation with PAF (100 nM). As shown in Fig. 1, Ze339 inhibited cysteinyl-LT synthesis with an IC_{50} of approximately 24 µg/mL (67.5 ± 9.0%) and a maximal inhibition at 40 µg/mL (87.9 ± 7.1%). Ze339 also inhibited LTB_4 synthesis, with an IC_{50} of approximately 24 µg/mL (48.8 ± 12.1%) and a maximal inhibition at 40 µg/mL (68.9 ± 12.4%). Higher concentrations of the drug did not further inhibit LT generation (data not shown). Since Ze339 blocked both cysteinyl-LT and LTB_4 synthesis, we performed the following experiments with cells specialised in the generation of either cysteinyl-LTs (eosinophils) or LTB_4 (neutrophils).

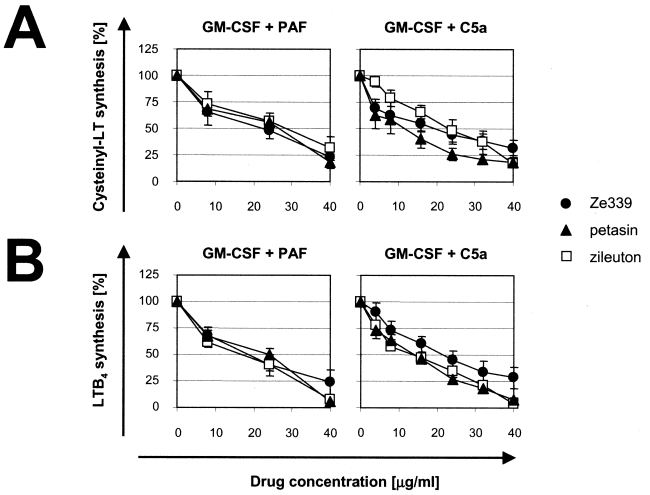

Fig. 2. Ze339 and petasin inhibited PAF- and C5a-induced LT synthesis in a dose-dependent manner in GM-CSF-primed eosinophils (A) and neutrophils (B). As a control, the 5-LO inhibitor zileuton was used. No significant differences between the compounds were found. Maximal LT synthesis in independent experiments ranged between 300–3,000 pg/mL for cysteinyl-LTs in eosinophils and 7,500–99,500 pg/mL for LTB_4 in neutrophils. Inhibition of LT synthesis is expressed as percent related to control cells stimulated without inhibitor. Values are means ± SEM of four to eight independent experiments ($*P < 0.05$).

3.2. Inhibition of eosinophil cysteinyl-LT and neutrophil LTB_4 synthesis by Ze339, petasin, and zileuton

Purified eosinophils and neutrophils were incubated with increasing concentrations of Ze339 and petasin. Zileuton, a commercially available 5-LO inhibitor, was used as positive control at the same concentrations. Eosinophils (1×10^6/mL) or neutrophils (1×10^7/mL) were then primed with GM-CSF and stimulated with PAF or C5a (no significant LT synthesis was observed using unprimed eosinophils or neutrophils; data not shown). As shown in Fig. 2, all compounds inhibited PAF- as well as C5a-mediated LT synthesis in GM-CSF-primed cells in a dose-dependent manner. Inhibitor concentrations above 40 µg/mL did not further inhibit the generation of LTs in either eosinophils or neutrophils (data not shown). No significant differences were observed among the blocking compounds inhibiting cysteinyl-LT generation in eosinophils and LTB_4 generation in neutrophils, respectively (IC_{50} was ≤ 24 µg/mL in each case). At a concentration of 40 µg/mL, cysteinyl-LT and LTB_4 synthesis inhibition ranged between 68–94% in C5a- or PAF-stimulated granulocytes.

3.3. Suppression of PAF- and C5a-mediated increases in $[Ca^{2+}]_i$ by Ze339 and petasin, but not by zileuton

PAF- and C5a-induced $[Ca^{2+}]_i$ changes were monitored over 250 sec. Cells were from different donors for each drug tracing, and maximal $[Ca^{2+}]_i$ changes ranged between 130 and 240 nM. As shown in Fig. 3A and Table 1, pretreatment with 16 µg/mL of Ze339 or petasin completely blocked

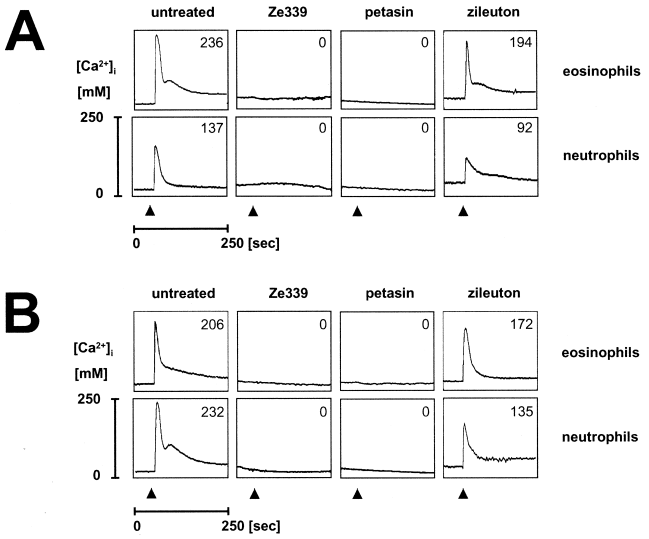

Fig. 3. Ze339 and petasin, but not zileuton, inhibited PAF- (A) or C5a- (B) induced increases in $[Ca^{2+}]_i$ in eosinophils and neutrophils. At 16 μg/mL, Ze339 and petasin completely abrogated the calcium signal. PAF or C5a was added where indicated (▲). The tracings are representative of four independent experiments.

PAF-induced increases in $[Ca^{2+}]_i$ in eosinophils and neutrophils. In contrast, PAF-induced $[Ca^{2+}]_i$ mobilisation was not significantly reduced by zileuton in either eosinophils or neutrophils. Similar results were obtained in C5a-stimulated eosinophils and neutrophils (Fig. 3B and Table 2) and PAF-stimulated macrophages (data not shown). These results suggest that Ze339 and petasin block signal transduction pathways initiated by G protein-coupled receptors at the level of or proximal to the release of calcium from intracellular stores. In contrast, the 5-LO inhibitor zileuton does not have this capacity.

4. Discussion

Eosinophils and neutrophils are important inflammatory effector cells that contribute to tissue damage due to the release of toxic mediators in allergic and infectious diseases. Besides the generation of toxic molecules, both cell types appear to amplify inflammatory responses by generating cytokines, chemokines, and lipid mediators. To improve the clinical situation of patients where activated granulocytes are part of the pathogenic process, blocking of either degranulation or generation of inflammatory mediators seems to be a reasonable strategy. Indeed, for patients with an accumulation of eosinophils in bronchial asthma [39,40] or neutrophils in chronic obstructive pulmonary disease (COPD) [23], blocking of granulocyte-derived LTs has been shown to be beneficial. There are two strategies to block LTs: (a) by synthesis blockers, such as 5-LO [39] or FLAP [41] inhibitors; or (b) by LT receptor antagonists [40].

In this study, we investigated the effect of Ze339 from *petasites hybridus* in two *in vitro* models of granulocyte activation. To determine whether petasin is relevant for the pharmacological activity of Ze339, we simultaneously analysed the effects of both compounds in the same *in vitro* models of LT synthesis. In both eosinophilic and neutrophilic granulocytes, priming of cells was performed by short-term incubation with GM-CSF. It has previously been shown that IL-3 and GM-CSF strongly enhance the response of granulocytes to different agonists [7–14]. After optimal stimulation with PAF or C5a, primed granulocytes generated large amounts of LTs. In both cell types, priming with GM-CSF was absolutely essential, since PAF or C5a alone did not significantly increase LT synthesis. Ze339 inhibited LT synthesis in stimulated macrophages, eosinophils, and neutrophils. The pharmacological activity of

Table 1
PAF-mediated increases in $[Ca^{2+}]_i$ are significantly inhibited by Ze339 and petasin, but not zileuton

Eosinophils			
Concentration	Ze339	Petasin	Zileuton
0	100	100	100
4 μg/mL	53.9 ± 6.2*	73.0 ± 1.3*	93.7 ± 2.6
8 μg/mL	30.2 ± 9.8*	58.3 ± 1.5*	78.3 ± 9.8
16 μg/mL	1.4 ± 1.4*	1.1 ± 1.1*	70.8 ± 8.3
Neutrophils			
Concentration	Ze339	Petasin	Zileuton
0	100	100	100
4 μg/mL	42.5 ± 5.8*	75.3 ± 2.0	78.1 ± 7.8
8 μg/mL	18.0 ± 2.0*	43.2 ± 3.7*	72.1 ± 8.5
16 μg/mL	0.8 ± 0.8*	1.1 ± 1.1*	59.4 ± 9.9

Experiments were performed as described in Materials and Methods and run as shown in Fig. 3A. The values represent percentages of the original peak calcium levels and are means ± SEM of four independent experiments. At 16 μg/mL, Ze339 and petasin, but not zileuton, completely blocked ligand-induced calcium mobilization.

* $P < 0.05$.

Table 2
C5a-mediated increases in $[Ca^{2+}]_i$ are significantly inhibited by Ze339 and petasin, but not zileuton

Eosinophils			
Concentration	Ze339	Petasin	Zileuton
0	100	100	100
4 μg/mL	63.1 ± 2.4*	59.6 ± 10.5*	87.0 ± 3.0
8 μg/mL	37.7 ± 7.5*	24.9 ± 6.1*	80.5 ± 1.9
16 μg/mL	0.9 ± 0.9*	0.7 ± 0.7*	62.6 ± 5.1
Neutrophils			
Concentration	Ze339	Petasin	Zileuton
0	100	100	100
4 μg/mL	56.1 ± 5.9*	65.5 ± 9.3	85.4 ± 8.1
8 μg/mL	37.4 ± 6.0*	36.7 ± 1.9*	64.3 ± 7.4
16 μg/mL	0.7 ± 0.7*	1.1 ± 1.1*	52.1 ± 2.2

Experiments were performed as described in Materials and Methods and run as shown in Fig. 3B. The values represent percentages of the original peak calcium levels and are means ± SEM of four independent experiments. At 16 μg/mL, Ze339 and petasin, but not zileuton, completely blocked ligand-induced calcium mobilization.
* $P < 0.05$.

Ze339 and petasin appeared to be in the same range as observed using the known 5-LO blocker zileuton [39].

In order to partially understand the mode of actions of Ze339 and petasin, we analysed PAF- and C5a-mediated increases in $[Ca^{2+}]_i$, an indirect marker for ligand-induced phospholipase C_β activation [42,43]. In addition, increases in $[Ca^{2+}]_i$ are required for activation of both $cPLA_2$ and 5-LO. Although Ze339, petasin, and zileuton blocked PAF- and C5a-induced LT production, only Ze339 and petasin, but not zileuton, significantly reduced increases in $[Ca^{2+}]_i$ induced by these two ligands in both eosinophils and neutrophils. This suggests that the calcium-dependent enzymes $cPLA_2$ and 5-LO, which are absolutely required for LT synthesis, are not sufficiently activated in the presence of adequate concentrations of Ze339 or petasin.

Moreover, $cPLA_2$ is suggested not only to be activated by increases in $[Ca^{2+}]_i$, but also by serine/threonine kinases of the MAPK pathway [44,45]. Binding of IL-3/GM-CSF to their receptor induces "priming" of granulocytes by activating the common β-subunit of their respective receptors [46]. Although this "priming" seems to be insufficient to reach the required threshold to induce functional responses over the MAPK pathway [47], G protein-coupled receptors might increase the activation of the MAPK pathway via protein kinase C acting on Ras [48] or Raf [49], resulting in an increased LT production in granulocytes [47]. In such a scenario, a drug that acts at the level of or proximal to phospholipase C_β (e.g. Ze339 or petasin) would block $cPLA_2$ 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.

The exact molecular targets of Ze339 remain to be identified. Moreover, it is unclear whether Ze339 has similar effects in other inflammatory effector cells of allergic reactions, such as mast cells. Clearly, Ze339-mediated reduced mast cell LT production would suggest an even higher anti-allergic and/or anti-inflammatory potential. The discovery that Ze339 and petasin suppress ligand-induced increases in $[Ca^{2+}]_i$ implies that these drugs may also block other calcium-dependent pathways, such as degranulation mechanisms.

Acknowledgments

This work was supported by grants from Max Zeller Söhne AG, Herbal Remedies, Romanshorn, Switzerland, and the Swiss National Science Foundation (Grant No. 31-58916.99).

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