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# The effect of sesquiterpene lactones on the release of human neutrophil elastase

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**Abstract**

Sesquiterpene lactones (SLs) are natural products responsible for the anti-inflammatory activity of a variety of medicinal plants, mainly from the Asteraceae family. Here, we investigated whether they also influence the process of exocytosis of pro-inflammatory enzymes, such as the human neutrophil elastase (HNE). Altogether, eight structurally different SLs from the eudesmanolide, guaianolide, pseudoguaianolide, and germacranolide type were studied. Neutrophils were isolated from fresh human blood. After pre-incubation with different concentrations of the respective SL and cytochalasin B, the exocytosis of elastase was initiated either by platelet activating factor or *N*-formyl-methionyl-leucyl-phenylalanine. Inhibition of HNE release was measured by *p*-nitroaniline formation. The SLs exhibited an inhibitory effect on elastase release from neutrophils challenged either by platelet activating factor or *N*-formyl-methionyl-leucyl-phenylalanine. Concentration–response curves were recorded and the $IC_{50}$ values ranged from 2 to 30 μM. Studies on isolated HNE showed that a selective direct inhibition on HNE can be excluded. Interestingly, the inhibitory activity did not correlate with the number of α,β-unsaturated carbonyl functions. The structure–activity relationship and the molecular mechanism are discussed.


*Keywords:* Sesquiterpene lactones; Neutrophil granulocytes; Human neutrophil elastase release; PAF; fMLP; Anti-inflammatory activity

## 1. Introduction

Neutrophil granulocytes possess an anti-infectious as well as a pro-inflammatory function. Primarily they prevent the growth of microbial pathogens, such as bacteria, fungi, viruses, and tumor cells. This ability is due to a number of specific activities, including adherence to blood vessel walls, transmigration into tissues, chemotaxis, degranulation and release of pro-inflammatory mediators, and phagocytosis. Therefore, lack of neutrophils and their dysfunction can have severe clinical consequences (neutropenia). However, intense stimulation of neutrophil granulocytes by pro-inflammatory cytokines and a dysfunction of the regulatory mechanisms, such as tachyphylaxis to pro-inflammatory mediators and apoptosis, can also cause severe tissue injury. As neutrophils are relatively unselective in their destroying ability by attacking invading microorganisms as well as healthy host tissue, they become the predominant contributor to tissue injury and play an important role in the pathogenesis of chronic inflammatory diseases [1–3].

Neutrophil functions are initiated by cell surface receptors of chemoattractants, such as the bacterial peptide *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), the platelet activating factor (PAF), or interleukin-8 (IL-8), resulting in exocytosis of granules with proteolytic enzymes, e.g. elastase and collagenase, or in the release of reactive oxygen species [3].

Elastase (HNE, IEC 3.4.21.37) is the major proteinase within the azurophilic granules of the human neutrophils and has the ability to degrade a wide variety of extracellular macromolecules, including elastin, fibronectin,

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*Abbreviations:* HNE, human neutrophil elastase; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; PAF, platelet activating factor; SLs, sesquiterpene lactones; SAAVNA, *N*-succinyl-L-alanyl-L-alanyl-L-valine-*p*-nitroanilide.

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and proteoglycans as well as plasma proteins, like immunoglobulines, clotting factors, and complement factors. HNE can also modulate the function of other inflammatory cells, such as lymphocyte activation and platelet aggregation as well as the influx of neutrophils, into the sites of inflammation by stimulating the secretion of granulocyte macrophage stimulating factor (GM-CSF), IL-6 and IL-8, from epithelial cells. Moreover, HNE can also modulate the function of other inflammatory cells, such as inducing lymphocyte activation and platelet aggregation [4]. Under normal physiological conditions, HNE is controlled by endogenous proteinase inhibitors. However, intense neutrophil infiltration results in an imbalance between the amount of HNE and its endogenous inhibitors. Accumulation of this enzyme can then cause abnormal degradation of healthy tissue resulting in the development of chronic inflammatory diseases, such as rheumatoid arthritis, pulmonary emphysema, adult respiratory distress syndrome (ARDS), cystic fibrosis, and delayed wound healing [5]. Therefore, looking for compounds which directly inhibit HNE or its release from human neutrophils is a major task in the screening for anti-inflammatory drugs.

Recently, we reported on the ability of some SLs, natural compounds from medicinal plants of the Asteraceae family, to inhibit the catalytic activity of HNE at low concentrations [6]. In addition, in numerous *in vitro* and *in vivo* studies it was proven that SLs modulate many inflammatory processes, such as the exocytosis of cathepsin G and acid phosphatase from the azurophilic granules of rat polymorphonuclear leukocytes and the release of histamine from mast cells and serotonine from blood platelets as well as the rat paw and mouse ear edema [7–10]. They inhibit the 5-lipoxygenase and leukotriene $C_4$ synthase in human blood cells [11]. Interestingly, it was shown for some SLs that they also possess apoptotic effects which can also be advantageous in inflammatory tissues by eliminating non-functional cells [12,13]. We could show that they possess cytokine suppressing properties by inhibiting the transcription factors NF-κB and NF-AT [10,14–16]. In continuation of our research on their anti-inflammatory activities we studied the ability of eight SLs of the eudesmanolide, guaianolide, pseudoguaianolide, and germacranolide type to inhibit the release of HNE from human neutrophil granulocytes.

## 2. Materials and methods

### 2.1. Material

The eight SLs were isolated from different Asteraceae species as listed in Table 1 [17–20]. Ten millimoles stock solutions were prepared in DMSO and then diluted with 10% DMSO to the final concentrations. The elastase substrate *N*-succinyl-L-alanyl-L-alanyl-L-valine-*p*-nitroanilide (SAAVNA) and cytochalasin B were purchased from Bachem Feinchemikalien AG. fMLP and PAF were from Sigma, Ficoll-Paque and Dextran T-500 from Amersham Pharmacia Biotech AB. The natural elastase inhibitor α1-anti-trypsin as well as the SLs, parthenolide and alantolactone/isoalantolactone, were purchased from Aldrich Chemicals. The absorbance was measured using a Shimadzu UV-Vis double-beam recording spectrometer. The following stock solutions were used for preparing the test solutions: cytochalasin B, 0.965 mg/mL in 20% DMSO; SAAVNA, 300 mM in DMSO; fMLP, 10 mM in EtOH; PAF, 10 mM in EtOH; and PBS with 0.9 mM $CaCl_2$, 0.49 mM $MgCl_2$, and 2.5% BSA to dilute the SAAVNA, PAF, and fMLP working solutions.

### 2.2. Neutrophil isolation

Purified human neutrophils were isolated from heparinized blood from healthy adult volunteers using a Ficoll-Paque gradient. Remaining erythrocytes were lysed by hypotonic shock. The resultant pellet was suspended at a concentration of $10 \times 10^6$–$30 \times 10^6$ cells/mL in PBS containing $Ca^{2+}$/$Mg^{2+}$ and 2.5% BSA (for details see [3]).

Table 1
Effects of sesquiterpene lactones **1**–**8** on the elastase release from human neutrophils

| | Substance | PAF | SD | fMLP | SD | Origin |
|---|---|---|---|---|---|---|
| **1** | Molephantin | 2.56 | 0.17 | 2.92 | 0.40 | *Elephantopus mollis* [17] |
| **2** | 4β,15-Epoxy-miller-9*E*-enolide | 2.90 | 0.24 | 2.90 | 0.24 | *Milleria quinqueflora* [18] |
| **3** | Parthenolide | 4.16 | 0.06 | 5.11 | 0.29 | Aldrich Chemicals |
| **4** | Scandenolide | 6.78 | 0.56 | 9.13 | 0.59 | *Mikania micrantha*[a] |
| **5** | 11α,13-Dihydrohelenalin methacrylate | 7.93 | 0.37 | 6.85 | 0.09 | *Arnica montana*[b] |
| **6** | 11α,13-Dihydrohelenalin acetate | 9.78 | 0.34 | 8.21 | 0.34 | *Arnica montana*[b] |
| **7** | Thieleanin | 10.90 | 1.18 | 12.09 | 1.06 | *Decachaeta thieleana*[c] |
| **8** | Alantolactone/isoalantolactone 3:1 | 29.39 | 1.73 | 24.09 | 1.13 | Aldrich Chemicals |
| | α1-AT | 10.49 | 1.10 | 11.88 | 0.73 | |

Given are the $IC_{50}$ values and the standard deviations of the investigated sesquiterpene lactones (μM) and the control substance α1-AT (μg/mL) as well as the name and the origin of the sesquiterpene lactones.

[a] Isolation: current thesis B. Siedle, identification [20].

[b] Isolation procedure described in Ref. [19].

[c] R. Murillo, V. Castro, A.J. Garcia-Piñeres, C.A. Klaas, I. Merfort, unpublished.

### 2.3. Elastase release assay

The elastase assay using SAAVNA as a substrate was conducted according to Ref. [3]. Briefly, the release of the enzyme elastase is quantified by the photometric measurement of *p*-nitroaniline (pNA), the colored product of the cleavage of SAAVNA by elastase. The neutrophils were incubated with the respective test compound at different concentrations and cytochalasin B (5 μg/mL) in the incubation medium PBS with $Ca^{2+}/Mg^{2+}$, 2.5% BSA, and SAAVNA (0.2 mM final concentration) for 5 min at 37°. The reaction was started by adding the stimulus, either 0.1 mM of PAF or fMLP, and the tubes were incubated for 10 min at 37°. Citric acid was added to stop the reaction. The samples were centrifuged and the absorbance of the supernatant was measured at 405 nm.

In our experience, the effect of DMSO, giving a final concentration of 0.1% had no significant effect on the neutrophils (data not shown) and did not appear to compromise the integrity of the results from the assay.

### 2.4. Inhibition assay of the isolated elastase

The inhibition assay was conducted according to Ref. [3]. Briefly, the human neutrophils were isolated as described earlier. The cells were diluted using PBS with $Ca^{2+}/Mg^{2+}$ and 2.5% BSA to a concentration of $1.5 \times 10^6$–$5 \times 10^6$ cells/mL. They were activated by the addition of cytochalasin B (5 μg/mL) and then incubated for 10 min at 37°. Addition of PAF (0.1 μM) initiated the release of elastase. After incubation (10 min) the exocytosis was stopped by centrifugation. The elastase-containing supernatant was mixed with the highest concentration of the respective SL used in the elastase release assay. The background reference samples were inactivated by adding citric acid (2%) and the reaction was initiated by the addition of SAAVNA solution at 37° for 30 min and was stopped by adding citric acid (2%). The absorbance of each sample was measured at 405 nm.

### 2.5. Cytotoxicity assay

Effects of SLs on cell viability were studied on human neutrophils, isolated as described earlier. Cells were suspended in PBS (having $Ca^{2+}/Mg^{2+}$ and 2.5% BSA) and incubated for 15 min at 37° with the respective SL in PBS. Viability of cells was checked by trypan blue exclusion.

### 2.6. Statistical analysis

All assays were performed at least three times with duplicate samples. Inhibition rates were calculated in percent to control without inhibitor. The results of each series are expressed as the mean values ± SD. Statistical analyses were performed using the paired Student's *t*-test. For determination of $IC_{50}$ values, log concentrations, and linear response data were analyzed by non-linear curve fitting using the program ORIGIN 7.0 (OriginLab Corporation).

## 3. Results

### 3.1. Effects on elastase release in human neutrophils

SLs **1**–**8** (for structures see Fig. 1) were studied for their inhibition on elastase release from neutrophils which were isolated from fresh human blood. Either PAF or fMLP were used to initiate the exocytosis of elastase. The amount of released enzyme was quantified by photometric measurement of the colored product pNA, that is formed by proteolytic cleavage of the oligopeptide SAAVNA. Concentration–response curves were recorded and $IC_{50}$ values were calculated by non-linear curve fitting (see Fig. 2). All SLs suppressed the PAF as well as the fMLP-mediated elastase release in a dose-dependent manner at micromolar concentrations. The calculated $IC_{50}$ values ranged from 2 to 30 μM (see Table 1).

The SLs exhibited a non-selective inhibitory effect on elastase released from neutrophils challenged by the two stimuli. The most active substance was the germacranolide molephantin (**1**), isolated from *Elephantopus mollis*, with an $IC_{50(PAF)}$ of 2.56 and an $IC_{50(fMLP)}$ of 2.92 μM. The other germacranolides (**2**, **3**, and **4**) inhibited the enzyme release in low micromolar concentrations ($IC_{50(PAF)}$: 2.9–6.8/ $IC_{50(fMLP)}$: 2.9–9.1 μM) as well. The substances with a pseudoguaianolide (**5** and **6**) and a guaianolide skeleton (**7**) exhibited slightly higher $IC_{50}$ values (**5**: 7.9/6.9; **6**: 9.8/ 8.2 μM). The mixture of the eudesmanolides (**8**, ratio 3:1) was less active with an $IC_{50}$ value of 29.4/24.1 μM. The eudesmanolides alantolactone and isoalantolactone only differ in the position of the double bond which does not influence their reactivity and are, therefore, regarded as one substance (**8**). The physiological peptidic elastase inhibitor α1-anti-trypsin was used as a positive control and showed a half-maximal inhibitory effect at 10–12 μM.

### 3.2. Effect on isolated elastase

In order to study whether the above observed effect is due to a direct attack on HNE, the highest concentration of each SL was tested for inhibition of the isolated enzyme. Most of the investigated compounds (**1**–**3**, **5**–**7**) did not significantly inhibit elastase. Inhibition values were in the range of −2 to 2%. This is in accordance with our previous investigations showing that HNE is not a direct target for SLs [6]. Only scandenolide (**4**) slightly inhibited the enzyme (25% inhibition at 20 μM). The least active eudesmanolide (**8**) even showed a slight activation of the enzyme activity (10% at 75 μM). From these findings it can be deduced that the observed effect on elastase release from neutrophils is not due to a direct inhibition of the enzyme. Therefore, it is highly likely

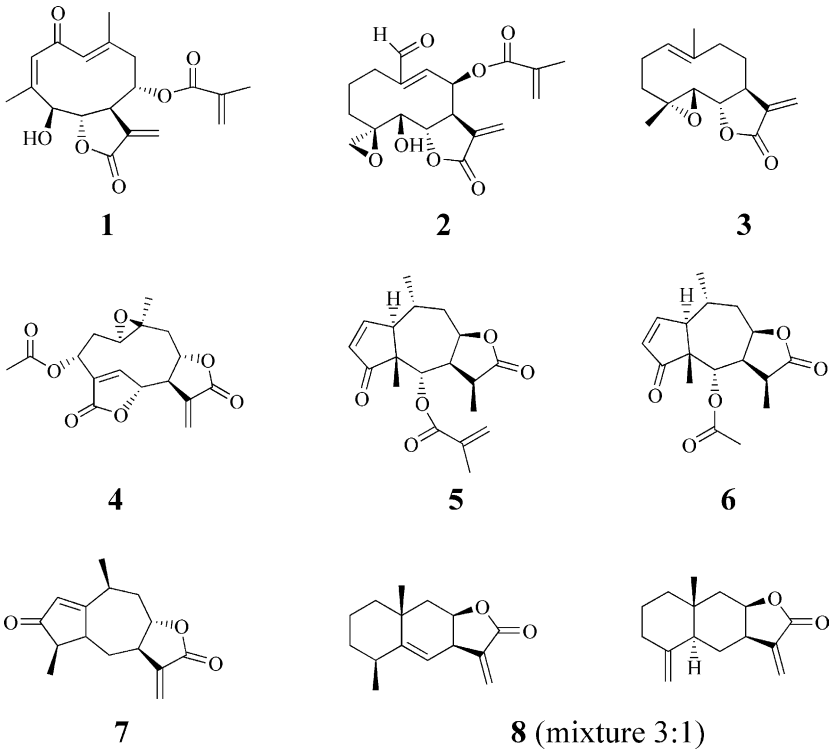


Fig. 1. Structures of the investigated sesquiterpene lactones.

that the signal transduction pathway leading to elastase release is mainly affected.

### 3.3. Cytotoxicity of SLs

To evaluate the cytotoxic potential of the SLs, the effect of the highest concentrations used in the elastase release assay was studied on viability of human neutrophils. At concentrations between 7.5 μM (**1**) and 75 μM (**8**) no cytotoxic effect could be observed using trypan blue as staining reagent. Therefore, it can be concluded that the $IC_{50}$ values reported here are not influenced by cytotoxicity.

## 4. Discussion

Dysregulated neutrophil recruitment and activation results in severe damage of adjacent normal tissues which can maintain inflammation processes. It is, therefore, of great interest to develop effective compounds which stop the release of tissue destroying enzymes from granulocytes, such as elastase, collagenase, cathepsin G, or myeloperoxidase, or the production of reactive oxygen derivatives. Compounds which possess this membrane stabilizing effect have the advantage of targeting several inflammatory mediators. This beneficial property can be applied to SLs, natural compounds being the constituents of many plants used in traditional medicine.

Here, we prove that they are able to inhibit the elastase release from neutrophil granulocytes in low micromolar concentrations. A direct attack as well as any cytotoxic effects could be excluded with the exception of compounds **4** and **8** which showed a slight, but neglectable, effect on isolated HNE. SLs **1–3** were the most active compounds in the elastase release assay with $IC_{50}$ values of <5 μM. Whereas compounds **1** and **2** are so-called bifunctional by possessing two α,β-unsaturated carbonyl structure elements, which have often been proven as an essential prerequisite for a strong anti-inflammatory activity [8,15,21], parthenolide (**3**) can be regarded as a kind of prodrug. It was shown that in acidic medium the epoxide structure of this germacrolide can form a cationic intermediate which easily reacts with nucleophiles [22]. Interestingly, all these compounds with very high activity belong to the germacranolide subfamily of SLs, which are characterized by a 10-membered ring system with a great conformational flexibility. The other active compounds with an $IC_{50}$ value between 5 and 12 μM are either monofunctional SLs lacking the α-methylene-γ-butyrolactone structure, like the dihydrohelenalin derivatives **5** and **6**, or bifunctional SLs with a rather rigid shape, like scandenolide (**4**), a germacranolide that looses its flexibility because of the epoxide and the additional lactone structure, and the guaianolide thieleanin (**7**). The least active eudesmanolide mixture (**8**) is monofunctional as well, but is characterized by a rather flat shape whereas the other structures are more or less angled. Thus, a certain flexibility or angle in the molecular structure might be important for the inhibitory activity. The pseudoguaianolides **5** and **6** serve special interest. Despite missing the

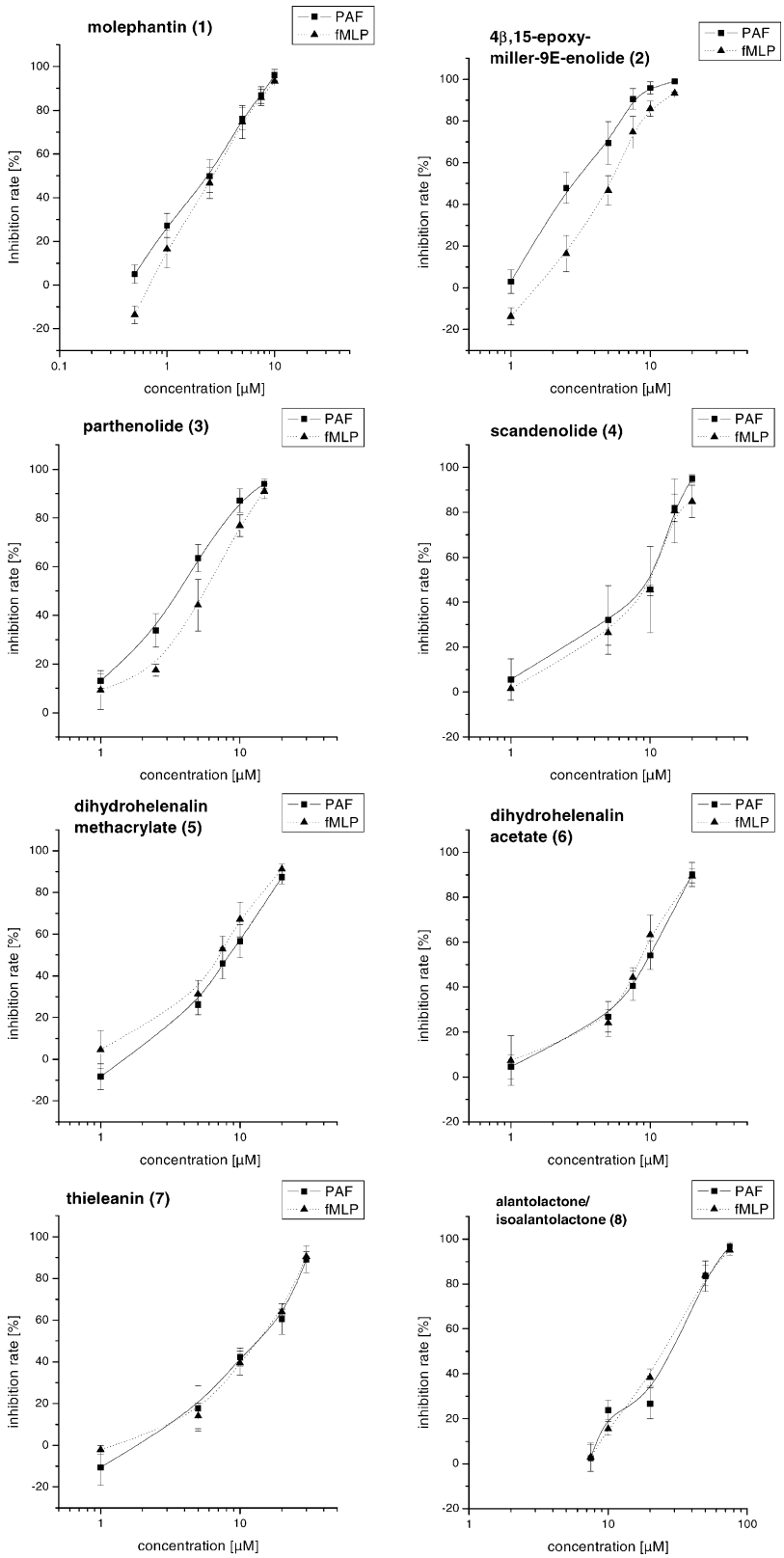


Fig. 2. Effects of sesquiterpene lactones **1–8** on elastase release from human neutrophils. For experimental conditions see Section 2.

α-methylene-γ-lactone structural element they show a comparable inhibitory activity to the compounds with this structural feature. This is in accordance with the studies of Hall *et al*. [7] who investigated 11,13-dihydropseudoguaianolides for inhibition of cathepsin G release from neutrophils and revealed good inhibitory activity for these compounds. Therefore, the assumption of Groenewegen *et al*. [23] that anti-secretory properties of SLs are only due

to the presence of the α-methylene-γ-lactone unit is not valid anymore. The occurrence of this structure element is not a prerequisite for the inhibitory activity of SLs on enzyme release.

Although the anti-secretory activity of SLs has been investigated by several groups, no examinations concerning the molecular mechanism of action have been undertaken yet. In previous studies we could show that inhibition of the transcription factor NF-κB plays an important role in SLs anti-inflammatory properties [14–16,24]. Moreover, it was shown that also downstream events, such as IL-8 release from human neutrophils, were prevented by the SL parthenolide [25]. However, it can be excluded that under the conditions of our assay, inhibition of elastase release is mediated by influence on the transcription factor NF-κB and downstream events, such as IL-8 release. A decrease in the cellular elastase concentration due to inhibition of NF-κB would only be detectable after several hours of incubation, because the protein synthesis has to be initiated. Here, we study the decrease in elastase release already after 15 min. Therefore, here short-term signal transduction mechanisms have to be considered in the regulation of exocytosis.

In this study we used two different stimuli, PAF and fMLP, to gain first insights which pathways may be affected by elastase. Both stimuli bind to G-protein-coupled receptors and activate distinct and separate effector pathways. fMLP is a formylated bacterial tripeptide which is a strong activator of human neutrophils. Exposure to fMLP results in a broad range of rapid functional responses, including actin assembly, adherence, calcium influx, chemokinesis, chemotaxis, superoxide production, and granule enzyme release [26]. These cellular responses are mediated by activation of several pathways. On the one hand, the second messenger system of phospholipase C is activated with subsequent generation of inositol triphosphate ($IP_3$) and diacylglycerol (DAG) and increase of the intracellular $Ca^{2+}$ concentrations. On the other hand, the Ras protein is activated and two cascades of protein kinases subsequently lead to the activation of the two MAPKs, p38 and ERK1/ERK2 (p42/44). Stimulation with the physiological phospholipide PAF results in minor utilization of these intracellular pathways, the p42/44 pathway is only slightly affected, but the p38 MAPK pathway is strongly activated [26,27]. All these activation pathways finally result in the release of proteolytic enzymes, like elastase, and the oxidative burst, that means the release of reactive oxygen species.

Because of the non-selective inhibition of the PAF respectively fMLP-induced elastase release SLs could affect the p38 MAPK pathway, in which both stimuli are involved. This theory is supported by the investigations of Hwang *et al.* [28], who showed that treatment of macrophage RAW 264.7 cells with parthenolide induced a dramatical decrease of tyrosine phosphorylation of MAPKs. Recently, parthenolide was investigated for its influence on the LPS-induced maturation of dendritic cells [29]. It could be proven that this SL prevents p38 MAPK activation by inhibiting the IL-1 receptor/TLR pathway at the level of MAPKKK or further upstream. However, it was also shown that the SL parthenolide does not influence LPS-induced phosphorylation of p38 MAPK in HeLa cells [30] and that this SL is an inhibitor of LPS-induced phosphorylation of p42/44 MAPK in rat primary microglial cells [31]. Further studies are necessary to clarify whether these discrepancies are due to the different cell types which were used in the assays. Moreover, it has to be discussed whether elevated levels of cyclic adenosine monophosphate, which have been induced by SLs, stabilize lysosomal membranes thus inhibiting the release of various enzymes [7].

Though the molecular mechanism by which SLs inhibit the exocytosis of degrading enzymes, such as the elastase, is not yet clear and can only be discussed, our studies extend our knowledge on the anti-inflammatory activity of these very interesting natural compounds, which are the effective principle of many medicinal plants used in traditional medicine for the treatment of inflammations. Inhibition of the release of tissue degrading enzymes, such as HNE, has beneficial effects in inflammatory processes, because inflammation is attacked at an early phase and tissue destroying processes are interrupted.

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