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Natural Compounds as Inhibitors of Human Neutrophil Elastase

Abstract

The imbalance between human neutrophil elastase (HNE) and endogenous serine proteinase inhibitors is considered to cause a variety of HNE-mediated inflammatory disorders. Thus, HNE has been the object of intensive research to find potent inhibitors that target its destructive and pro-inflammatory action. This review focuses on natural compounds which have been demonstrated to inhibit the enzyme itself or its release from neutrophils. Some of the natural compounds discussed here may serve as lead structures suitable to be used for the development of semi-synthetic inhibitors, but up to now none has been found active enough to be directly used in therapy.

Key words
Human neutrophil elastase · biological role · involvement in diseases · assays · natural compound inhibitors · direct inhibition · inhibition of release

Introduction

Elastases are a group of serine proteases that possess the ability to cleave the important connective tissue protein elastin, which is widely distributed in vertebrate tissue, and is particularly abundant in the lung, arteries, skin, and ligaments. These proteases include the neutrophil elastase (NE), also known as leukocyte elastase, the pancreatic elastase (PE), the macrophage elastase (MMP-12) and the fibroblast elastase [1], [2], [3]. There has been increasing interest in elastases in recent years because of their possible involvement in diseases of the connective tissues. This review highlights the biological role of human neutrophil elastase (HNE), its involvement in diseases, and subsequently its role as a target for drug research concentrating on biogenic compounds.

Human Neutrophil Elastase

HNE (E.C. 3.4.21.37) is a 30-kD glycoprotein chymotrypsin-like serine proteinase and is synthesized as zymogen that requires two separate amino-terminal processing steps to become active [4]. The enzyme has a specificity for small hydrophobic amino acids. The potent catalytic activity is facilitated by a catalytic triad that is conserved in all serine proteases and consists of His, Asp, and Ser residues forming a charge relay system. During proteolysis the side chain of the peptide is located in the S1 specificity pocket. Its backbone carbonyl is placed in the 'oxyanion hole' and forms hydrogen bonds with the NH of Gly193 and Ser195, thus stabilizing the charge transition state [1]. Along with cathepsin G and proteinase 3 (PR3), HNE is stored at high concentrations (5 mM) in its active form in azurophil granules of neutrophils. These granules undergo differential exocytosis following neutrophil activation upon exposure to various cytokines and chemoattractants. This includes TNF-α, IL-8, C5a, LPS, PAF (platelet-activating factor), PMA (phorbol-12-myristate-13-acetate),

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and fMLP (a tripeptide derived from the bacterial wall, *N*-formylmethionyl-leucyl-phenylalanine). Whereas the exact mechanism of granule exocytosis is not yet fully understood, it is known that ligation of cell-surface receptors (such as β_2-integrins) activates a cascade of cytoplasmic signalling molecules, triggering the influx of calcium ions and subsequently the exocytosis of HNE. HNE is released into the extracellular space, but remains mostly bound to the neutrophil plasma membrane [5].

The biological role of HNE

The primary role of the intracellular HNE appears to be the proteolysis of foreign proteins (e.g., from bacteria) during phagocytosis by neutrophils. This antibacterial role is directed towards Gram-negative but not Gram-positive bacteria. HNE acts via catalytic proteolysis of specific Gram-negative outer wall proteins (OmpA) (e.g., from *E. coli*) or cleaves bacterial virulence factors [4]. Consequently, it was shown that mice deficient in HNE have impaired survival rates in the presence of Gram-negative infections [6]. HNE also contributes to protection against certain fungal infections [4].

In addition to its intracellular antibacterial activity, HNE also exhibits extracellular antibacterial effects. It has been shown that the bacterial flagellin, a virulence factor with a pro-inflammatory effect on epithelial cells, was cleaved, thereby abrogating the ability of flagellin to induce a pro-inflammatory host response.

Because of its broad substrate specificity, extracellular HNE degrades a variety of host proteins (such as extracellular macromolecules, including elastin, fibronectin and proteoglycans) as well as plasma proteins, like immunoglobulines, clotting factors, and complement factors. Due to this property, secreted HNE can degrade local ECM (extracellular matrix) proteins, 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. Nevertheless, there has been little evidence that HNE plays the role of a path-clearer for neutrophil migration [7]. Additionally, HNE may also function as a negative regulator of inflammation by degrading various pro-inflammatory cytokines, such as IL-1, TNF-α and IL-6 [8], [9].

Under normal physiological conditions, HNE is controlled by serpins, which are endogenous serine proteinase inhibitors that trap it and distort the catalytic site. These inhibitors include α1-antitrypsin, elafin, and the secretory leukocyte proteinase inhibitor (SLPI) [4], [9], [10]. They can lose their protective role for several reasons. Large quantities of oxidants and proteases released by leukocytes that are recruited to the site of inflammation can inactivate these endogenous inhibitors. Moreover, tight adhesion of neutrophils to the ECM leads to the compartmentalization of the released proteases between the neutrophil and the ECM, thereby excluding the large, circulating protease inhibitors. Tight binding of extracellular HNE to the cell membrane can render it inaccessible to circulating endogenous inhibitors [4]. Altogether, the imbalance between HNE and its inhibitors caused by these events provokes severe tissue injuries resulting in a variety of diseases.

HNE and its involvement in diseases

It has become clear that serine proteases, such as HNE, have an important regulatory role in the local inflammatory response. Thus, its dysregulation resulting in its accumulation can be involved in the development of chronic inflammatory diseases, such as rheumatoid arthritis, pulmonary emphysema, adult respiratory distress syndrome (ARDS), cystic fibrosis, COPD, asthma, and delayed wound healing [5], [11], [12], [13].

Emphysema, due to α_1-antitrypsin deficiency, and cystic fibrosis (CF) belong to the most common lethal hereditary disorders in white populations. The block of α_1-antitrypsin processing in hepatocytes significantly reduces levels of circulating α_1-antitrypsin, which may lead to emphysema due to insufficient protection of the lower respiratory tract from HNE, permitting progressive destruction of the alveoli [14]. In CF, impaired mucocilliary clearance leads to chronic bacterial infections and subsequent vigorous influx of neutrophils in the airways. High levels of HNE are released and induce progressive proteolytic impairment of multiple defense pathways leading to endobronchial obstruction and airway wall destruction [14]. Studies also indicate that HNE contributes to chronic inflammatory airway diseases by inducing mucin production in airway epithelial cells. HNE is often associated with ARDS, but observational studies of humans do not yet convincingly demonstrate the role for HNE [5].

Rheumatoid arthritis is characterized by the infiltration of mononuclear cells and neutrophils into the synovial tissue with pathological degradation of cartilage and the formation of pannus tissue resulting in joint destruction. HNE has a high affinity for cartilage tissue and can degrade major cartilage tissue components [11]. Besides HNE, reactive oxygen species are released from neutrophils by which endogenous inhibitors (e.g., α_1-antitrypsin) are oxidized and prevented from binding to elastase [15].

According to other studies, uncontrolled elastase activity may be also implicated in delayed wound healing. Knock-out mice for the secretory leukoprotease inhibitor (SLPI) showed increased elastase activity and tissue inflammation, along with delayed closure of cutaneous wounds [5].

Moreover, active HNE is known to be present in psoriatic lesions. It induces keratinocyte hyperproliferation by proteolytic activation of an EGFR signalling pathway involving TGF-α [16], [17].

Laboratory research and clinical findings have indicated that a deficiency in α1-antitrypsin is associated with increased risk of various cancer diseases and that raised levels of elastase might promote the development, invasion, and metastasis of many cancers. It is postulated that HNE might degrade the intercellular matrix barrier, and might contribute to cancer development through the TNF-α signalling pathway [18]. Accordingly, inhibition of HNE has been shown to suppress the development of skin tumours in hairless mice [19].

Previous work has demonstrated that decreases in skin elasticity, accompanied by increases in the tortuosity of elastic fibers, are early events in wrinkle formation especially after UV exposure

[2]. Furthermore, neutrophils are supposed to participate in the process of photoageing of human skin as they infiltrate the skin and release enzymatically active HNE [20]. Additionally, keratinocytes and fibroblasts also produce elastases, but to a lower extent [2], [21].

In summary, the increasing knowledge of the role of HNE in these various diseases has considerably increased the interest in discovering potent HNE inhibitors in the last years.

Assays to Screen for HNE Inhibitors

There are several possibilities to detect either the effect on elastase or on its release. Very often the assay described by [22] is performed. Here the isolated HNE and MeO-Suc-Ala-Ala-Pro-Val-*p*NA as substrate are used and the release of *p*-nitroaniline is measured photometrically. However, despite the simplicity of the assay, reproducibility is not always guaranteed as the enzyme is very sensitive to the pH, electrolytes, and also the substrate [23], [24], [25]. For screening of elastase inhibitors on a large scale, a chemical array was developed [26] using a composite microarray. After a proteinase film had formed, the chemicals and the mixed chromogenic solutions were subsequently printed at the same sites. Chromogenic differences demonstrate whether chemicals inhibit enzymatic activity. HNE inhibition can also be studied using the surface plasmon resonance (SPR) technology where HNE is immobilized to sensor chips [27]. Another approach is the use of the catalytic domain of HNE which is expressed in *E. coli* [28]. An additional, but more expensive possibility is the use of an elastase ELISA [29]. These assays only allow the study of the direct effect on HNE. In contrast, the neutrophil multitarget functional bioassay described by Johansson et al. [30] and optimized for using microplates [31] can be used to screen compounds for their inhibitory activity on HNE release from neutrophils as well as the direct effect on isolated HNE. There are two problems related to this assay. Granulocytes are very easily upregulated during isolation from fresh blood, but this can be significantly reduced by collecting blood from volunteers with an empty stomach and who have drunk a cup of water half an hour before blood collection, because these volunteers have less lipids in their blood (personal communication T. Simmet, University of Ulm). Additionally, the substrate used is also cleaved by other serine proteases (such as proteinase 2) because of their structural and functional similarity. This cleavage may be avoided by serpin-derived fluorogenic substrates [32].

Natural Compounds as HNE Inhibitors

Recombinant endogenous elastase inhibitors [such as recombinant secretory leukocyte proteinase inhibitor, α1-proteinase inhibitor and skin-derived antileukoprotease as well as a recombinant-derived protein from the inter-alpha-trypsin inhibitor (EPI-HNE-4)], have become already available [33]. Interestingly, sivelestat (for structure see Fig. **1**) is the only synthetic inhibitor that reached the clinical market [13]. This intravenously effective, reversible and competitive HNE inhibitor with an IC_{50} value of 44 nM has been proven to exhibit protective effects against various causes of lung injuries [34]. In the field of synthetic small molecules acyl-enzyme-inhibitors and transitionstate inhibitors are considered to be the most promising approaches [34]. In addition to that, natural compounds may also be an interesting source to screen for inhibitors which either directly inhibit the enzyme or its release from neutrophils.

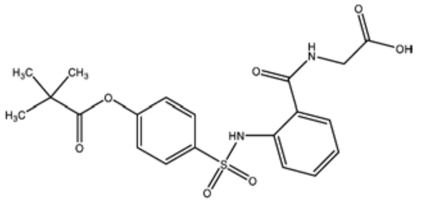


Fig. **1** Structure of sivelestat which has reached the clinical market.

Phenolics, such as flavonoids, tannins and further cinnamic acid derivatives have been reported as **direct HNE inhibitors**. Different flavonoids, including aglyca and glycosides, were investigated for their HNE inhibitory activity by Melzig et al. [35] (see Fig. **2A** and Fig. **2B**). Compounds with a catecholic structural element – two neighbouring phenolic hydroxy groups – showed remarkable activity. In the group of flavones, luteolin (with a catecholic structure in ring B) exhibited an IC_{50} value of 12.5 μM. This activity is significantly decreased by methylation of one of the phenolic groups as in diosmetin (IC_{50} = 83 μM). Interestingly, chrysin (without any catecholic structural element) showed even higher activity (IC_{50} = 6.7 μM). The inhibitory activity may also be dependent on the double-bond C-3/C-2 in the flavonoid C-ring, as the investigated flavanones naringenin and eriocitrin only show a weak activity. Glycosylation also seems to influence HNE inhibitory activity. Different glycosides of quercetin differ from one another (IC_{50} range 0.3 – 11.1 μM) and from the aglycone quercetin (IC_{50} = 2.4 μM) in their inhibitory activity (see Fig. **2A** and Fig. **2B**). Some of these flavonoids and two anthocyanidins were also studied by Sartor et al. [36]. These authors obtained mostly higher IC_{50} values (see Fig. **2A** and Fig. **2B**).

However, it has to be kept in mind that flavonoids are highly metabolized during oral application [37], [38] and that some possible metabolites (such as 4-methylcatechol, 4-hydroxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid) exhibited a very low activity in the assay (IC_{50} range from 135 μM to > 400 μM, see Fig. **2A** and Fig. **2B**) [35]. Therefore, the *in vitro* studies mentioned above may be of limited therapeutic relevance and it is questionable that flavonoids may be orally active principles in a *Drosera* extract used to treat cough [39]. Moreover, inhibition of HNE may not be reasonable as an approach for cough treatment in common cold because of the necessity of pathogen defense.

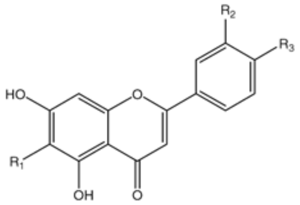
In contrast, external application of more lipophilic flavonoids, which does not have the problem of biodegradation, may have beneficial effects given that the inhibitory activity is sufficient. In this respect the chalcone phloretin, but not 3′-hydroxyphloretin from *Malus doumeri* may be used in the field of skin care (see Fig. **2A** and **2B**) [40].

A very low IC_{50} value of 0.4 μM was found for EGCG [(–)-epigallocatechin 3-gallate], the most abundant flavanol of green tea [41]. This concentration can be reached in the plasma of moderate green tea drinkers. Moreover, bioavailability was also proven after external application [41], [42]. In our lab EGCG achieved an

IC_{50} value of 25.3 μM [43]. This discrepancy may be explained by the different experimental conditions and the sensitivity of the enzyme (see also section on HNE release).

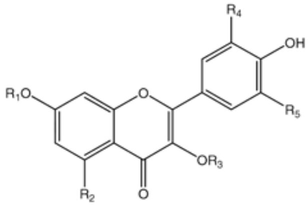
Another important class of phenolic plant compounds studied for the inhibition of HNE are the caffeic acid derivatives [35], [44], [45], [46] occurring ubiquitously in the plant kingdom (for investigated structures see Fig. **3A** and Fig. **3B**; Fig. **4A** and Fig. **4B**). Some of these substances with a catecholic structural element and lipophilic residues (such as bornylcinnamic acid ester derivatives (IC_{50} values 1.6 – 69 μM) [46], the *Cimicifuga* acids [45], and the triterpene esters from *Oenothera biennis* [44]) show remarkable activity in inhibiting HNE. Caffeic acid itself was weakly active in the assay (IC_{50} = 93 μM).

FLAVONES



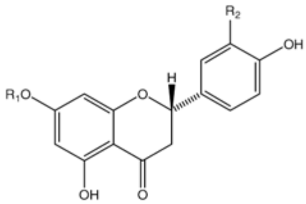
Compound	R_1	R_2	R_3	IC_{50} [µM] ref. in brackets
luteolin	H	OH	OH	12.0 [35]; >300 [36]
baicalein	OH	H	H	2.2 [35]; 25 [36]
chrysin	H	H	H	6.7 [35] no inhibition [36]
diosmetin	H	OH	OCH_3	83.0 [35]

FLAVONOLS



Compound	R_1	R_2	R_3	R_4	R_5	IC_{50} [µM] ref. in brackets
quercetin	H	OH	H	OH	H	2.4 [35]; 20 [36]
hyperoside	H	OH	galac	OH	H	0.3 [35]
quercitrin	H	OH	rham	OH	H	11.1 [35]
rutin	H	OH	rut	OH	H	9.8 [35] no inhibition [36]
isoquercitrin	H	OH	gluc	OH	H	1.4 [35]
kaempferol	H	OH	H	H	H	6.3 [35]; 5000 [36]
astragalin	H	OH	H	H	H	304 [35]
myricetin	H	OH	H	OH	OH	21.1 [35]; 4 [36]
rhamnetin	CH_3	OH	H	OH	H	18.3 [35]
morin	H	OH	H	H	OH 2'-OH	11.6 [35]; 4.5 [36]
fisetin	H	H	H	OH	H	16 [36]

FLAVANONES



Compound	R_1	R_2	IC_{50} [µM] ref. in brackets
naringenin	H	H	84 [35]
eriocitrin	rut	OH	>400 [35]

Fig. **2A** Flavonoids studied for their HNE inhibitory activity and the respective IC_{50} values.

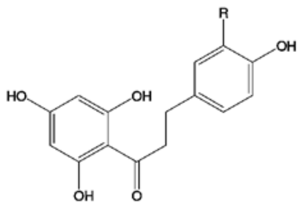
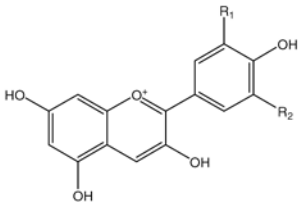
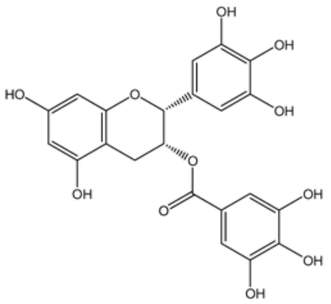
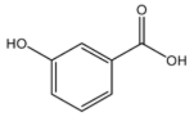
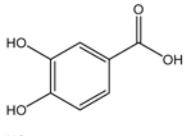
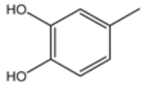
Compound			IC$_{50}$ [μM] ref. in brackets
DIHYDROCHALCONES			
			
	R		
phloretin	H		< 36.5 [40] no inhibition [36]
3'-hydroxyphloretin	OH		~ 100 [40]
ANTHOCYANIDINS			
	R$_1$	R$_2$	
delphinidin	OH	OH	12 [36]
pelargonidin	H	H	<3 [36]
FLAVANOLS			
(-) epigallocatechin-3-gallate (EGCG)			0.4 [41]; 25.3 [43]
URINARY METABOLITES OF FLAVONOIDS			
3-hydroxyphenylacetic acid			>400 [35]
3,4-dihydroxyphenylacetic acid			135 [35]
4-methylcatechol			>400 [35]

Fig. **2B** Flavonoids studied for their HNE inhibitory activity and the respective IC$_{50}$ values.

Ligand docking calculations were performed to investigate the mechanism of enzyme inhibition for the cinnamic acid derivatives [47]. It could be shown that interactions between the phenolic hydroxy groups and the enzyme's "oxyanion hole" (Ser195, Gly193) as well as with Cys191 and His195 of the catalytic triad are highly likely. The lipophilic residue is supposed to be located in the S1 specificity pocket of HNE. In contrast to flavonoids, caffeic acid esters have been shown to be resorbed

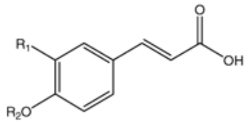
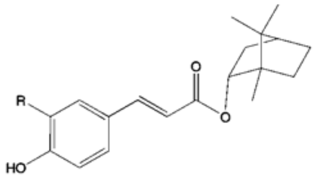
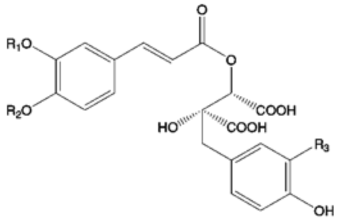
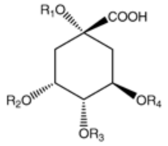
compound					IC$_{50}$ [µM] ref. in brackets
HYDROXYCINNAMIC ACID AND ITS ESTERS					
	R$_1$	R$_2$			
caffeic acid	OH	H			93 [35], [45], [46]; 475 [49]
isoferulic acid	OH	CH$_3$			> 1000 [35]; > 50 [45]
ferulic acid	OCH$_3$	H			> 1000 [35], [45], [46]
p-coumaric acid	H	H			> 1000 [35], [46]
BORNYL HYDROXYCINNAMIC ACID DERIVATIVES					
	R				
(-)-bornyl caffeate	OH				1.6 [35], [46]
(-)-bornyl coumarate	H				69 [46]
(-)-bornyl ferulate	OCH$_3$				78 [46]
CINNAMIC ACID DERIVATIVES from *Cimicifuga racemosa*					
	R$_1$	R$_2$	R$_3$		
fukinolic acid	H	H	OH		0.23 [45]
cimicifugic acid A	CH$_3$	H	OH		2.2 [45]
cimicifugic acid B	H	CH$_3$	OH		11.4 [45]
cimicifugic acid E	CH$_3$	H	H		> 50 [45]
cimicifugic acid F	H	CH$_3$	H		18 [45]
QUINIC ACID DERIVATIVES					
	R$_1$	R$_2$	R$_3$	R$_4$	
(-)-quinic acid	H	H	H	H	> 400 [35]
3-O-caffeoylquinic acid	H	caffeoyl	H	H	450 [35]
4-O-caffeoylquinic acid	H	H	caffeoyl	H	480 [35]
chlorogenic acid	H	H	H	caffeoyl	> 400 [35]
1,5-dicaffeoylquinic acid	caffeoyl	H	H	caffeoyl	151 [35]
3,5-dicaffeoylquinic acid	H	caffeoyl	H	caffeoyl	0.2 [35]

Fig. **3A** Caffeic acid derivatives studied for their HNE inhibitory activity and the respective IC$_{50}$ values.

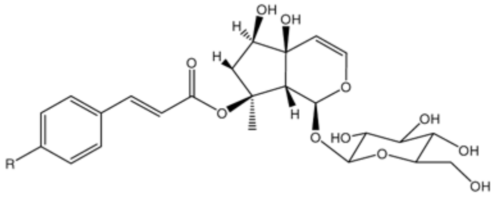
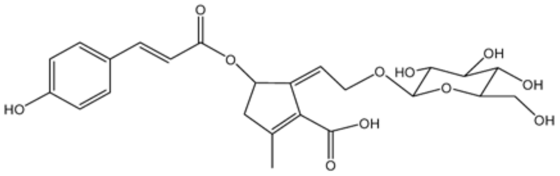
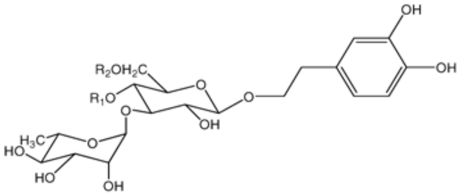
compound			IC_{50} [µM] ref. in brackets
IRIDOIDS from *Harpagophytum procumbens*			
	R		
harpagoside	H		>800 [49]
8-PCHG (8-*p*-coumaroylharpagide)	OH		331 [49]
pagoside from *Harpagophytum procumbens*			260 [49]
PHENYLETHANOID GLYCOSIDES from *Harpagophytum procumbens*			
	R_1	R_2	
acteoside	caffeoyl	H	>800 [49]
isoacteoside	H	caffeoyl	286 [49]
6`-O-acetylacteoside	caffeoyl	acetyl	70 [49]

Fig. **3B** Caffeic acid derivatives studied for their HNE inhibitory activity and the respective IC_{50} values.

after oral application. However, after *i. v.* administration in rats, the esters were rapidly transformed into the free caffeic acids [48]. Caffeic acid derivatives may contribute to the anti-inflammatory activity of traditionally used plant extracts by inhibition of HNE.

The ellagitannins agrimoniin and pedunculagin (for structures see Fig. **4B**) were proven to be potent HNE inhibitors. In one study ligand docking calculations revealed that inhibition may occur in an unspecific manner [43].

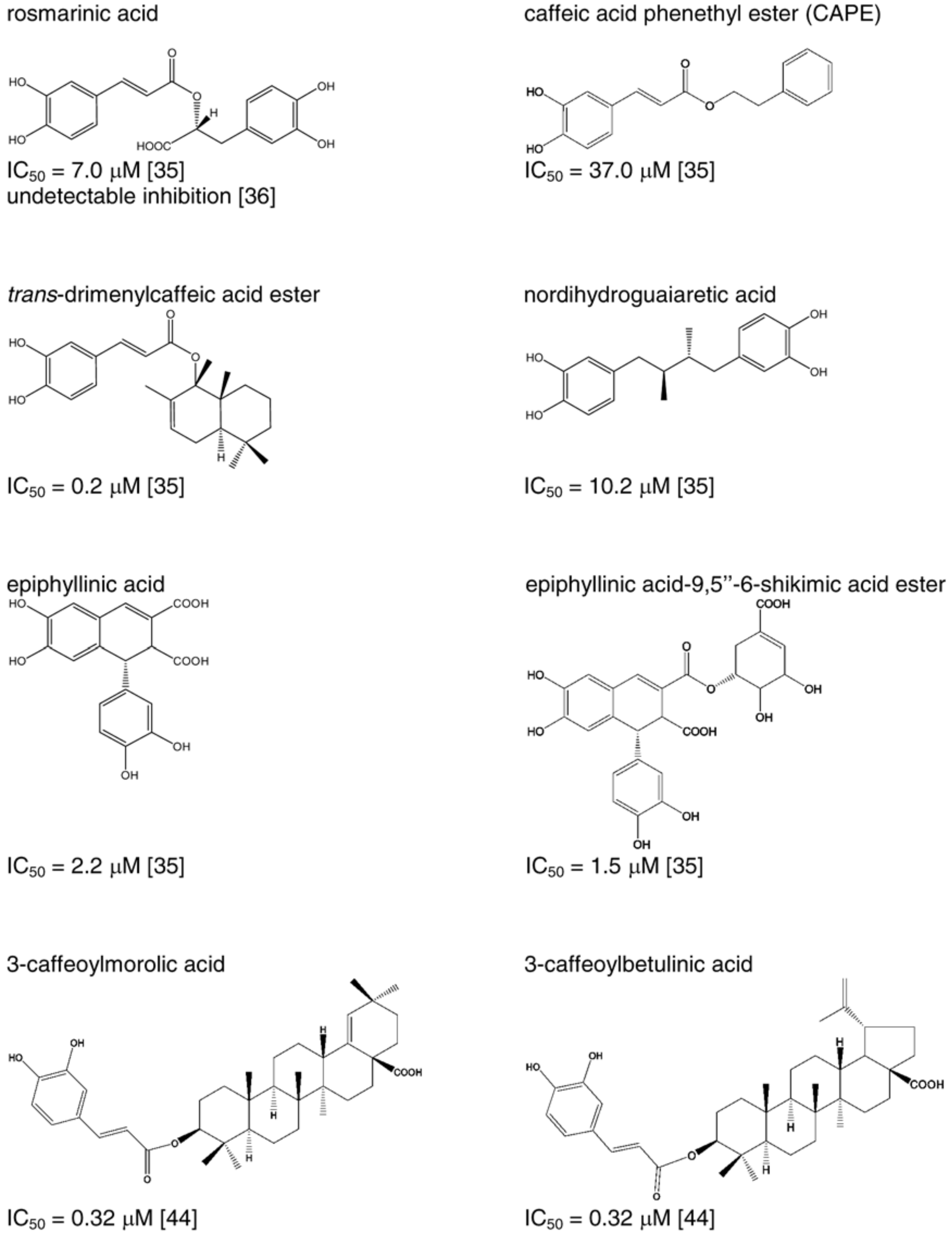
Compounds from devil's claw (*Harpagophytum procumbens*) (such as iridoids, pagoside and phenylethanoid glycosides) were investigated [49]. Only 6′-*O*-acetylacteoside gave an IC_{50} value beyond 100 µM (70 µM) (see Fig. **3A** and Fig. **3B**).

Concerning **terpenoids**, five monoterpenes from *Nigella sativa* have been analyzed for inhibition of HNE by Kacem and Meraihi [50]. IC_{50} values between 12 and 104 µM have been determined (see Fig. **5**). Interestingly, the phenolics thymol and carvacrol – structural isomers only differing in the OH position – vary in their inhibitory activity, indicating that the stereochemistry of thymol may be less favourable for interaction with HNE. In contrast to these results, Braga et al. excluded a direct inhibition of HNE by thymol up to a concentration of 133 µM, but only found inhibition of HNE release [51].

Seventeen sesquiterpene lactones (SLs) investigated in an *in vitro* assay mostly exhibited a very moderate inhibitory activity against HNE (see Fig. **6A**, Fig. **6B** and Fig. **6C**) [52]. Podachaenin was the most active compound with an IC_{50} value of 7 µM. The studied SLs (IC_{50} range 7 to > 200 µM) do not covalently bind to the amino acids of the catalytic triad, thus differing from other (semi)-synthetic elastase inhibitors with a lactone moiety. In contrast to most other biological activities of SLs, HNE inhibition is not mediated by α,β-unsaturated carbonyl functions. Ligand binding calculations have demonstrated that the occurrence of a carbonyl function together with a hydroxy group or two hydroxy groups in a certain distance from one another seem to be a prerequisite for the inhibitory activity of SLs. As revealed by the computer model, the activity of the examined SLs could not be explained by a uniform molecular inhibitory mechanism. It has been postulated that in addition to hydrogen bonds to the oxyanion hole, podachaenin is able to fill a major part of the furrow in which the peptidic substrate is normally bound. The ability of inhibiting HNE release by some SLs has also been tested, see Fig. Fig. **6A**, Fig. **6B** and Fig. **6C** [53].

The sesquiterpene acid dehydrocostic acid also inhibited HNE giving an IC_{50} of 43 μM [54]. As the corresponding illicic acid was not active in the assay (structures see Fig. **6A**), the authors assumed that the semiplanar olefinic ring system with exocyclic conjugation may be responsible for HNE inhibition.

Pentacyclic triterpenes showed HNE inhibitory activity with different strength. Lupeol, ursolic acid, oleanolic acid, and canophyllol were the most active with IC_{50} values at 1.9 μM, 4.4 μM, 6.4 and 2.5 μM, respectively (see Fig. **7**) [55], [56], [57]. Experiments with substrate oligopeptides possessing different chain lengths showed that the binding site of triterpenes is situated between the enzyme's S_3 and S_5 specificity pocket [57]. It appeared that HNE inhibition depends on the presence and the orientation of the two reactive groups (C-28-COOH and C-3-OH or C-3-O) in the tested molecules, distant from 10 – 12 Å, reacting with Arg217 in S_4 – S_5 subsites of the extended substrate-binding domain of HNE, and S_3, respectively. Furthermore, hydrophobic interactions with Phe192 in the S_3 specificity pocket are assumed [55], [57].



rosmarinic acid

IC_{50} = 7.0 μM [35]
undetectable inhibition [36]

caffeic acid phenethyl ester (CAPE)

IC_{50} = 37.0 μM [35]

trans-drimenylcaffeic acid ester

IC_{50} = 0.2 μM [35]

nordihydroguaiaretic acid

IC_{50} = 10.2 μM [35]

epiphyllinic acid

IC_{50} = 2.2 μM [35]

epiphyllinic acid-9,5''-6-shikimic acid ester

IC_{50} = 1.5 μM [35]

3-caffeoylmorolic acid

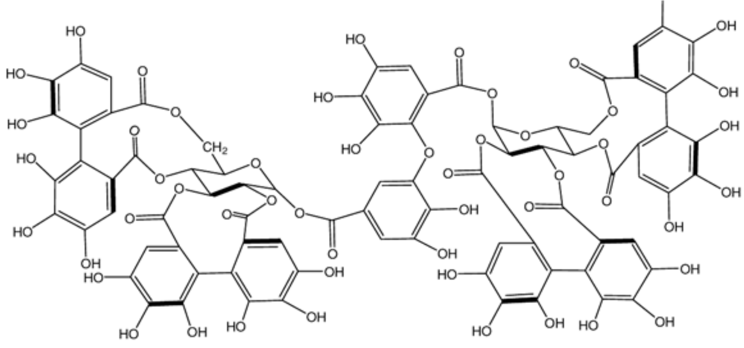
IC_{50} = 0.32 μM [44]

3-caffeoylbetulinic acid

IC_{50} = 0.32 μM [44]

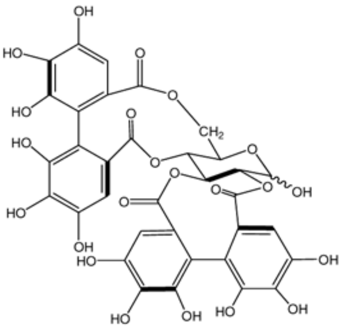
Fig. **4A** Miscellaneous phenolic compounds studied for their HNE inhibitory activity and the respective IC_{50} values (references given in brackets).

agrimoniin



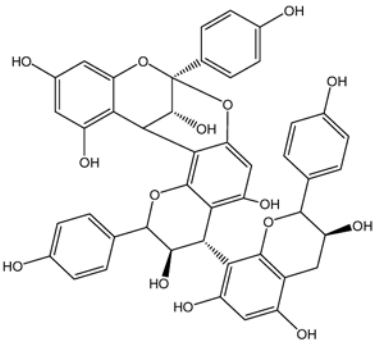
IC_{50} = 0.9 μM [43]

pedunculagin



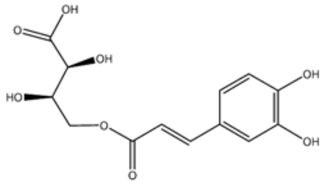
IC_{50} = 2.8 μM [43]

selligueain



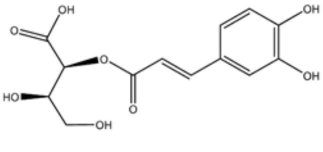
IC_{50} = 40 μM [69]

4-O-caffeoyl-L-threonic acid



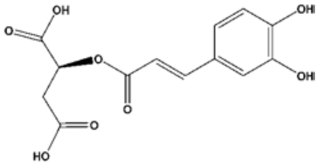
IC_{50} = 194 μM [35]

2-O-caffeoyl-L-threonic acid



IC_{50} = > 400 μM [35]

2-O-caffeoylmalic acid



IC_{50} = 33.7 μM [35]

Fig. **4B** Miscellaneous phenolic compounds studied for their HNE inhibitory activity and the respective IC_{50} values (references given in brackets).

For the first time Ashe et al. published that **fatty acids** are able to inhibit HNE [58]. They showed that trypsin, chymotrypsin and pancreatic elastase remain totally unaffected and that *cis*-unsaturation was a prerequisite. Later on, an additional study on their ability to inhibit proteolytic enzymes such as HNE and collagenase was reported, including 17 saturated and unsaturated fatty acids (see Fig. **8**) [59]. The saturated fatty acids with a chain length of 15 and more carbon atoms inhibited HNE to 50% at concentrations between 10 and 50 μM, indicating that a minimal chain length may be a prerequisite. Stearic acid was the most active substance in this test series of saturated fatty acids (IC_{50} = 10 μM). The unsaturated fatty acids exhibited IC_{50} values between 0.45 μM and 50 μM, whereas the polyunsaturated fatty acids (20 : 5 and 22 : 6) lack any inhibitory potential, indicating that a maximal degree of unsaturation should not be exceeded. The most active compound was erucic acid (22 : 1) with an IC_{50} value of 450 nM. Concerning the molecular inhibition mechanism, incorporation into the specificity pockets of the hydrophobic amino acids can be assumed. Fatty acids possibly occupy similar binding positions as shown for triterpenes. Tyagi et al. [60] proposed a role for at least one arginine residue in a hydrophobic environment in regulating binding and catalysis by HNE. Hence, inhibitors, such as oleic acid, which interact with both, should be especially potent and selective for HNE.

Based on these results a formulation of oleic acid with albumin was developed for the treatment of chronic wounds [61]. Albumin was used as a carrier for the hydrophobic oleic acid. Oleic

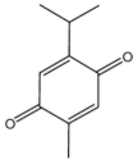
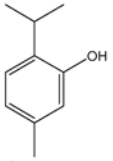
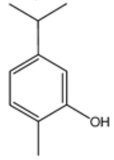
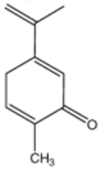
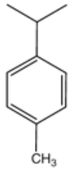
compound		IC_{50} [μM]	compound		IC_{50} [μM]
thymoquinone		30 [50]	thymol		104 [50]
carvacrol		12 [50]	carvone		14 [50]
cymene		25 [50]			

Fig. **5** Monoterpenes studied for their HNE inhibitory activity and the respective IC_{50} values (references given in brackets).

acid/albumin formulations with mole ratios of 100 : 1, 50 : 1, and 25 : 1 showed a strong inhibition of HNE with IC_{50} values at 0.029 – 0.049 μM. Albumin alone increased to a small extent the substrate conversion by HNE, which could be equalized by a higher concentration of inhibitor. The authors suppose that an increase of the albumin concentration may even have positive effects, since albumin level is decreased in chronic wounds. The formulation was still active (IC_{50} = 0.26 – 0.42 μM), even after being bound to derivatized cotton.

For natural and semi-synthetic sulfated **carbohydrates** HNE inhibitory properties have been described [62]. Their inhibitory activity is improved not only with increasing molecular weight and degree of sulfation, but depends also on their genuine polysaccharide structure. For β-1,3-glucan sulfate an IC_{50} of 18.1 nM was determined. For heparin, electrostatic interactions between the negatively charged sulfate groups of the molecule and the positively charged arginine residues on the surface of the enzyme are discussed. Heparin is also able to accelerate inhibition of HNE by mucus proteinase inhibitor, the predominant antielastase of lung secretions [63].

Recent studies from Spencer et al. [64] shed new light on the mechanism and structural requirements of HNE inhibition by heparin. According to their results heparin inhibits HNE by a tight-binding, hyperbolic, competitive mechanism. A minimum chain length of at least 12 – 14 saccharides is necessary for inhibition, after which inhibitory activity increases with chain length or molecular mass. All N- and O-sulfate groups, especially the N- or 6-O-sulfate groups, contribute to the inhibitory activity. Molecular docking simulations provided a plausible model for the size requirements, whereby positively charged regions at the end of the interdomain elastase fold are used by heparin to bridge the active site resulting in inhibition of HNE. Moreover, it was also reported that heparin inibited HNE release [65].

Previously, a **proteinaceous** inhibitor was isolated from *Tamarindus indica* seeds [66]. Depending on the analytical method the molecular weight of the molecule (PG50) differs from 14.9 kDa to 11.6 kDa. PG50 exhibited an IC_{50} value of 55.96 μg/mL against HNE, whereas no activity could be shown against porcine pancreatic elastase. Additionally, the inhibitor was also effective against HNE release induced by PAF or fMLP. At a concentration of 56 μg/mL HNE release was inhibited after stimulation with PAF to 44.6 % and with fMLP to 28.4 %.

Considering natural compounds **as inhibitors of HNE release** several **phenolics** have been reported. The flavonol quercetin (for structure see Fig. **2A**) was the first natural compound which was shown to inhibit the degranulation of neutrophils [67]. This compound inhibited the release of lactoferrin and HNE after stimulation with various stimuli at an IC_{50} value of 20 μM without influencing the viability of the cells. Degranulation was measured in a radioimmunoassay. Further studies on the mechanism were undertaken and it was concluded that quercetin influences the function of several neutrophil kinases by inhibiting their phosphorylation.

A standardized fraction of oligomeric proanthocyanidins (mean molecular weight 1100 ± 80 Da) from the seeds of *Vitis vinifera* was studied for its effect on cytochalasin B and calcium ionophore A23187 induced HNE release, and an IC_{50} of 5.4 μM was reported [68]. Quercetin and catechin were included in the study, but exhibited maximal inhibition (40 – 70 %) only at 30 μM. The authors speculated that the oligomeric catechins could exert a membrane-stabilizing effect by binding to the membrane surface phospholipids and preventing the fusion of the vesicles with the cytoplasma membrane.

The proanthocyanidin selligueain (structure in Fig. **4B**) from *Polypodium* species was tested for its inhibition on HNE release from leukocytes and an IC_{50} value of 40 μM was achieved. However, further experiments suggested that this compound is rather an enzyme inhibitor for HNE than for its release [69]. An unclear result was also obtained with the flavonol glycoside kaempferol 3-*O*-β-D-xylopyranosyl-(1→2)-β-D-arabinopyranoside which probably exerts a direct effect on the enzyme as well as on its release. However, these results may lack any therapeutic relevance, because of the doubtful bioavailability already discussed above.

Chalcones may also have a dual mechanism. Whereas phloretin (see Fig. **2B**) was shown to be a direct HNE inhibitor, viscolin (structure see Fig. **9**) proved to be an effective inhibitor of HNE release with an IC_{50} value of 9.48 μM (= 4.93 μg/mL) [70]. Further results indicated that this inhibition may be due to an elevation of cellular cAMP through inhibition of phosphodiesterase.

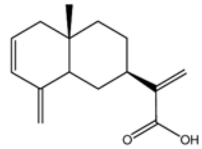
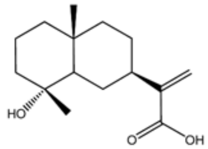
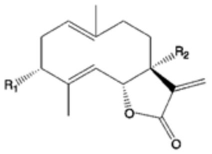
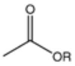
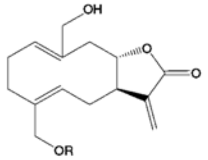
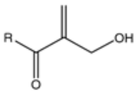
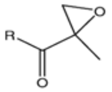
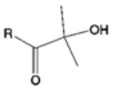
compound			direct inhibition IC_{50} [μM]	inhibition of HNE release IC_{50} [μM] PAF	IC_{50} [μM] fMLP
dehydrocostic acid			43 [54]		
ilicic acid			no inhibition [54]		
					
	R_1	R_2			
7-hydroxy-costunolide	H	OH	29 [52]		
3-acetoxy-costunolide		H	70 [52]		
					
	R				
15-(3`-hydroxy)-methacryloyloxy-micrantholide			15 [52]		
15-(2`,3`-epoxy)-isobutyryloxy-micrantholide			99 [52]		
15-(2`-hydroxy)-isobutyryloxy-micrantholide			33 [52]		

Fig. **6A** Sesquiterpenes studied for their inhibitory activity on HNE and on its release and the respective IC_{50} values (references given in brackets).

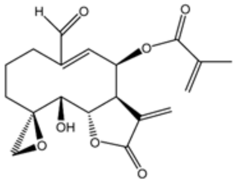
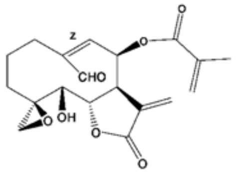
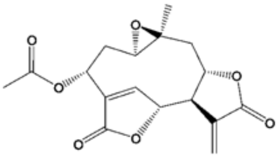
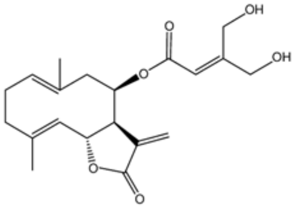
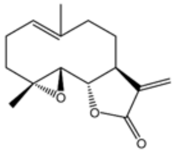
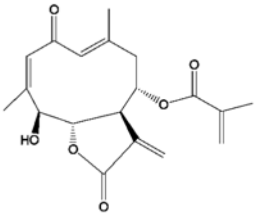
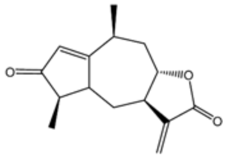
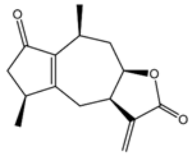
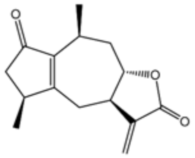
compound		direct inhibition IC_{50} [μM]	inhibition of HNE release	
			IC_{50} [μM] PAF	IC_{50} [μM] fMLP
4β-,15-epoxy-miller-9E-enolide		no inhibition	2.90 [53]	2.90 [53]
4β-,15-epoxy-miller-9Z-enolide		82 [52]		
scandenolide		91 [52]	6.78 [53]	9.13 [53]
eupatoriopikrin		144 [52]		
parthenolide		>100 [52]	4.16 [53]	5.11 [53]
molephantin		no inhibition	2.56 [53]	2.92 [53]
thieleanin		93 [52]	10.90 [53]	12.09 [53]
2-oxo-guai-1(5)-en-12,8α-olide		>200 [52]		
2-oxo-guai-1(5)-en-12,8β-olide		>200 [52]		

Fig. **6B** Sesquiterpenes studied for their inhibitory activity on HNE and on its release and the respective IC_{50} values (references given in brackets).

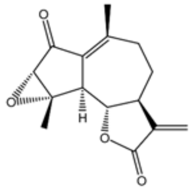
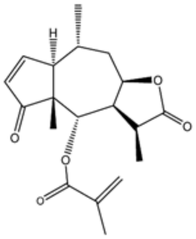
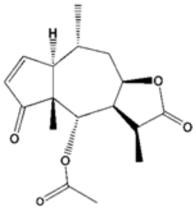
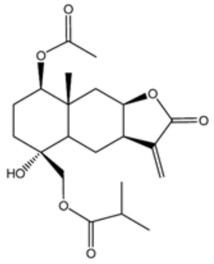
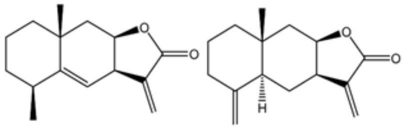
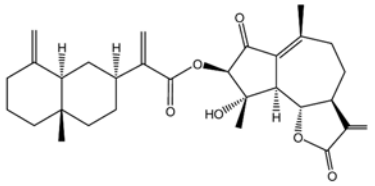
compound		direct inhibition IC_{50} [µM]	inhibition of HNE release	
			IC_{50} [µM] PAF	IC_{50} [µM] fMLP
eminensin		110 [52]		
11α,13-dihydrohelenalin-methacrylate		102 [52]	7.93 [53]	6.85 [53]
11α,13-dihydrohelenalin-acetate		>200 [52]	9.78 [53]	8.21 [53]
1ß-acetoxy-4α-hydroxy-15-isobutyryloxy-eudesma-11(13)-en-12,8ß-olide		>200 [52]		
alantolactone / isoalantolactone (3:1)		no inhibition	29.39 [53]	24.09 [53]
podachaenin		7 [52]		

Fig. **6C** Sesquiterpenes studied for their inhibitory activity on HNE and on its release and the respective IC_{50} values (references given in brackets).

Resveratrol, a natural phenol from red wine (for structure see Fig. **9**), was reported to inhibit HNE release from neutrophils induced by several stimuli [71]. Comparable IC_{50} values were obtained (fMLP: IC_{50} = 31 µM; complement factor C5a: IC_{50} = 41.6 µM; calcium ionophore A23187: IC_{50} = 37.7 µM) indicating a receptor-independent mechanism. The authors assumed an influence on protein tyrosine phosphorylation. Hrenn et al. revealed an IC_{50} of 12.0 µM for PAF-stimulated neutrophils [43]. As different IC_{50} values have often been reported in literature, no conclusion can be drawn, whether this result is due to the different stimulus or experimental design.

Different IC_{50} values have also been reported for the known tyrosine kinase inhibitor genistein (see Fig. **9**) for inhibition of HNE release. Tou et al. found an IC_{50} of 99 µM after stimulation with fMLP [72], whereas Hrenn et al. determined an IC_{50} value of 0.5 µM after PAF stimulation [43]. Discrepancies can also be found for EGCG (for structure see Fig. **2B**). An IC_{50} of 214 µM after

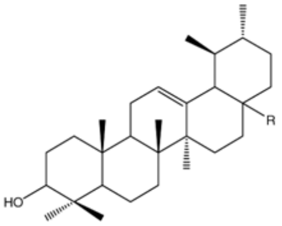
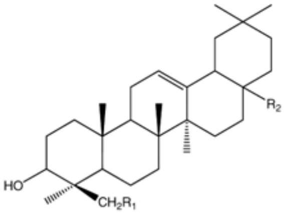
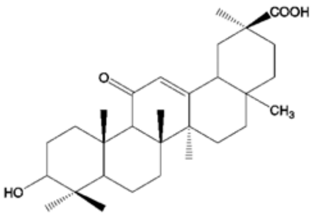
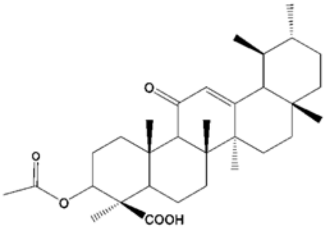
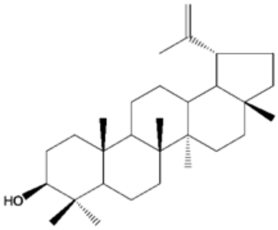
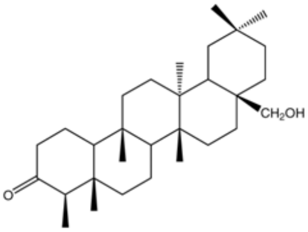
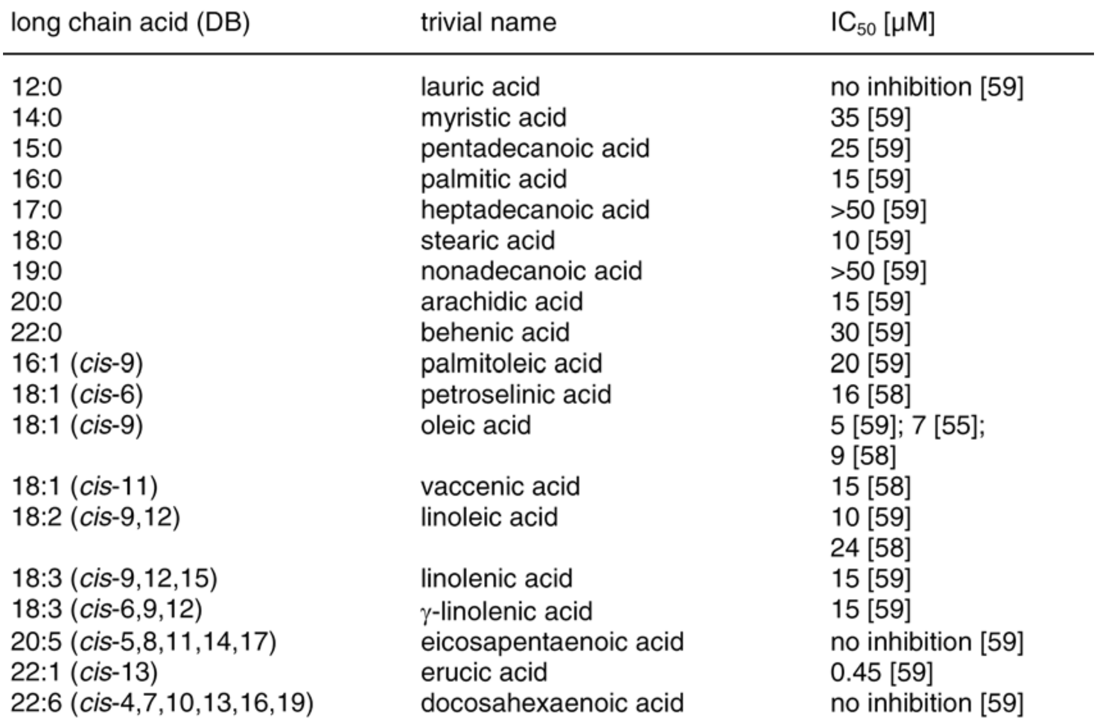
compound	structure		IC$_{50}$ [μM]
			
	R		
uvaol	CH_2OH		15.7 [57]
ursolic acid	COOH		4.4 [55]; 4.4 to 6.6 [57]; 0.9 to 2.4 [56]; 33.7 [62]
			
	R$_1$	R$_2$	
oleanolic acid	H	COOH	6.4 ± 1.0 [57]
erythrodiol	H	CH_2OH	17.3 ± 0.6 [57]
hederagenin	OH	COOH	62 ± 5.5 [57]
18 ß-glycyrrhetic acid			185 [57]
acetyl-11-keto-ß-boswellic acid (AKBA)			13.8 [56]
lupeol			1.9 [55]
canophyllol			2.5 [55]

Fig. **7** Triterpenes studied for their HNE inhibitory activity and the respective IC$_{50}$ values (references given in brackets).



long chain acid (DB)	trivial name	IC_{50} [µM]
12:0	lauric acid	no inhibition [59]
14:0	myristic acid	35 [59]
15:0	pentadecanoic acid	25 [59]
16:0	palmitic acid	15 [59]
17:0	heptadecanoic acid	>50 [59]
18:0	stearic acid	10 [59]
19:0	nonadecanoic acid	>50 [59]
20:0	arachidic acid	15 [59]
22:0	behenic acid	30 [59]
16:1 (*cis*-9)	palmitoleic acid	20 [59]
18:1 (*cis*-6)	petroselinic acid	16 [58]
18:1 (*cis*-9)	oleic acid	5 [59]; 7 [55]; 9 [58]
18:1 (*cis*-11)	vaccenic acid	15 [58]
18:2 (*cis*-9,12)	linoleic acid	10 [59] 24 [58]
18:3 (*cis*-9,12,15)	linolenic acid	15 [59]
18:3 (*cis*-6,9,12)	γ-linolenic acid	15 [59]
20:5 (*cis*-5,8,11,14,17)	eicosapentaenoic acid	no inhibition [59]
22:1 (*cis*-13)	erucic acid	0.45 [59]
22:6 (*cis*-4,7,10,13,16,19)	docosahexaenoic acid	no inhibition [59]

Fig. **8** Long-chain fatty acids studied for their HNE inhibitory activity and the respective IC_{50} values (references given in brackets).

fMLP stimulation was published by [72]. No inhibition on HNE release was detected by [43], but in this case concentrations higher than 200 µM were not tested because of the lack of therapeutic relevance.

Dicaffeoylquinic acid derivatives extracted from the Asteraceae *Phagnalon rupestre* inhibited HNE release from neutrophils after stimulation with TPA (12-*O*-tetradecanoylphorbol-13-acetate) at low micromolar concentrations (IC_{50} values between 4.8 and 10 µM) (for structures see Fig. **9**) [73]. No significant direct influence was observed on HNE. This result contrasts with investigations from Melzig et al. [35] in which 3,5-di-*O*-caffeoylquinic acid directly inhibited HNE to 50% at a concentration of 0.2 µM. No explanations can be given and further studies are necessary to clarify this discrepancy.

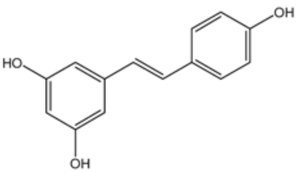
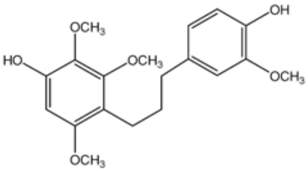
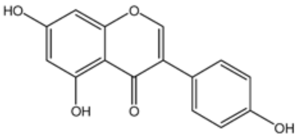
Three acylphloroglucinols, myrtucommulone and semimyrtucommulone from the leaves of myrtle (*Myrtus communis*) [74] and hyperforin from *Hypericum perforatum* [75], were studied for their ability to inhibit HNE release after stimulation with fMLP from neutrophils, and very low IC_{50} values (0.4 – 3.8 µM) were determined (see Fig. **9**). Further results revealed that the inhibitory activity may be explained by targeting components within G protein signalling cascades leading to a suppression of receptor-mediated Ca^{2+} mobilization [74], [75]. Direct inhibition was either excluded up to 3 µM or not investigated.

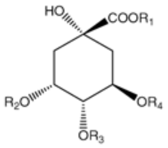
As already mentioned, contradictory results exist for the **terpenoid** thymol. Whereas Kacem and Meraihi [50] found a direct inhibition of HNE, Braga et al. reported an inhibition of release to 33.3% at a concentration of 133 µM (20 µg/mL) [51].

Sesquiterpene derivatives (for structures see Fig. **10**) from the sponge *Dysidea* spec. were shown to markedly inhibit HNE release after stimulation with fMLP [76]. Direct inhibition of the enzyme was excluded. IC_{50} values of 5.3 µM and 1.3 µM were obtained from bolinaquinone and the sesquiterpene aminoquinone dysidine, respectively. A mixture of the two diastereomers dysidenone A and B (1 : 1) was less active with an IC_{50} value of about 10 µM.

Eight structurally different SLs were studied for inhibition of HNE release. Compared to direct inhibition [52], only low concentrations were mostly needed to observe significant effects after PAF or fMLP stimulation (see Fig. **6A**, Fig. **6B** and Fig. **6C**). IC_{50} values ranged from 2 to 30 µM. The inhibitory activity did not correlate with the number of α,β-unsaturated carbonyl functions. Taking into consideration the 3-dimensional structure of the molecules a certain flexibility or angle in the molecular structure might be important for the inhibitory activity. As two different stimuli were used for the experiments which revealed similar results, it was discussed that SLs possibly influence the p38 MAP kinase pathways, that are activated by both stimuli.

The high potential of natural compounds is their role as **lead structures** which can be optimized concerning biological activity and/or bioavailability. The knowledge of structure-activity relationships of natural HNE inhibitors has been used and their structures have been modified in many respects. Thus, peptide sequences which fit the extended binding sites of HNE were covalently coupled to oleic acid [77]. These fatty acid peptide derivatives behave as competitive inhibitors towards HNE and also porcine pancreatic elastase. Modifications of the carboxylic end group of the peptide to an aldehyde further enhanced the inhibition capacity.

compound	structure	IC$_{50}$ [μM]
resveratrol		fMLP: 31 [71] C5a: 41.6 [71] A23187: 37.7 [71] PAF: 12.0 [43]
viscolin		9.5 [70]
genistein		0.5 [43] 99 [72]



	R$_1$	R$_2$	R$_3$	R$_4$	
3,5-di-O-caffeoyl-1-methyl-quinic acid	CH$_3$	caffeoyl	H	caffeoyl	8.9 [73]
3,5-di-O-caffeoyl quinic acid	H	caffeoyl	H	caffeoyl	9.9 [73]
4,5-di-O-caffeoyl quinic acid	H	H	caffeoyl	caffeoyl	4.8 [73]

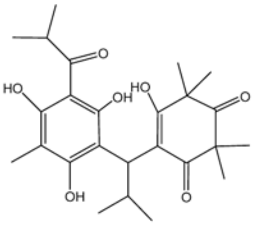
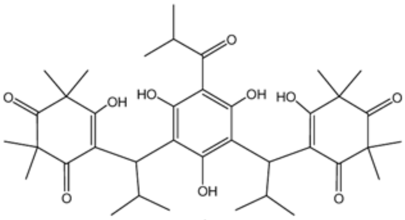
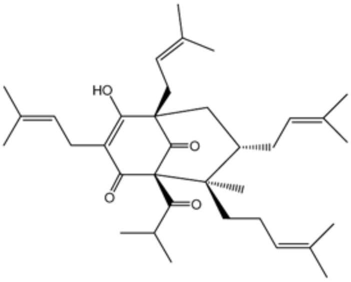
semimyrtucommulone S-MC		3.8 [74]
myrtucommulone MC		0.9 [74]
hyperforin		0.4 [75]

Fig. **9** Phenolics studied for inhibition of HNE release (references given in brackets).

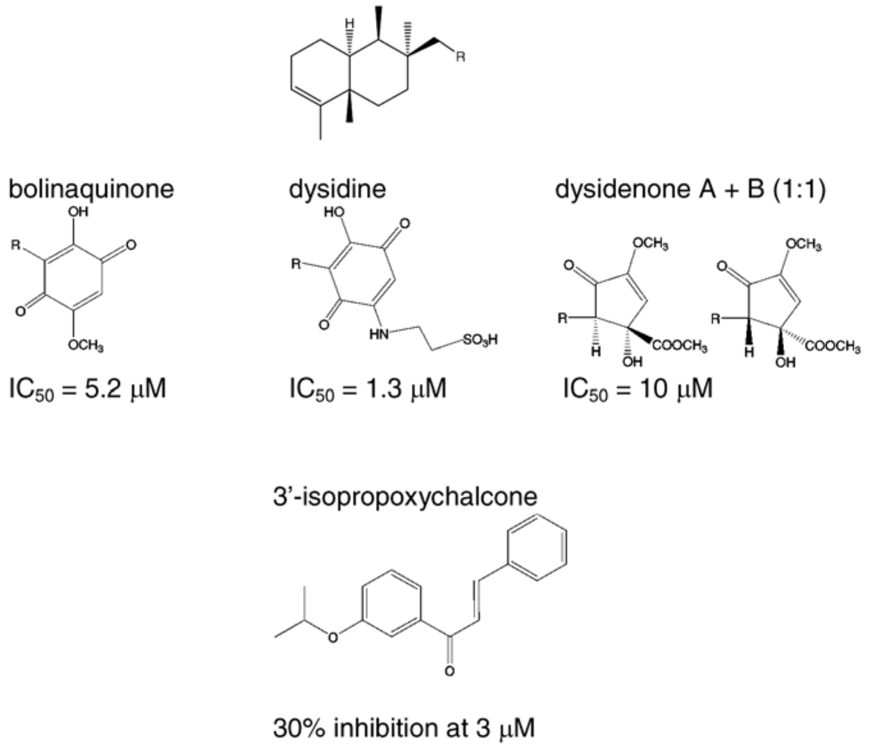


Fig. **10** Structures of the investigated sesquiterpene derivatives from *Dysidea* species and of 3′-isopropoxychalcone which inhibit HNE release.

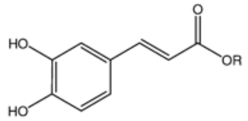
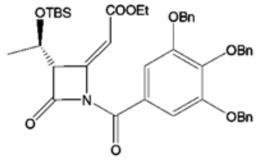
compound	R	IC$_{50}$ [µM]
		
n-octylcaffeic acid	n-octyl	1 [78]
n-hexyl-caffeic acid	n-hexyl	5 [78]
n-butylcaffeic acid	n-butyl	8 [78]
phenylpropylcaffeic acid ester	phenylpropyl	22 [78]
benzylcaffeic ester	benzyl	145 [78]
methylcaffeic acid ester	CH3	>400 [78]
ethylcaffeic ester	ethyl	>400 [78]
isopropylcaffeic acid ester	isopropyl	>400 [78]
tert-butylcaffeic acid ester	tert-butyl	>400 [78]
n-hexadecyl-/n-octadecylcaffeic acid ester		not determinable [78]
β-lactam with a galloyl moiety		0.7 [80]

Fig. **11** Semisynthetic compounds studied for their HNE inhibitory activity and the respective IC$_{50}$ values (references given in brackets).

A similar approach was conducted by Melzig et al. [78]. As caffeoyl derivatives with a lipophilic residue (see Fig. **3A** and Fig. **3B**) have been often proven to be potent HNE inhibitors his group tested semi-synthetic caffeic acid esters with a lipophilic alkoxy moiety. Dependent on the length and geometry of the chain as well as on the shape of the molecule inhibitory properties varied (see Fig. **11**). *n*-Octylcaffeic acid was the most active compound with an IC_{50} of 1 μM.

Based on the results of Sartor et al. [36], [41] that catechins with a galloyl group are powerful HNE inhibitors and of Knight et al. [79] that β-lactams can be developed as a general class of serine protease inhibitors, Dell'Aica et al. have synthesized and tested a number of monocyclic β-lactam derivatives with a galloyl-like group in different positions [80]. {3-[1-(*tert*-butyldimethylsiloxy)-ethyl]-4-oxo-1-[3,4,5-tris(benzyloxy)benzoyl]-azetidin-2-ylidene}-acetic acid ethyl ester (structure see Fig. **11**) turned out to be the most potent non-competetive inhibitor with an IC_{50} below micromolar concentrations and Ki = 0.7 μM.

A couple of coumarins and isocoumarins have been synthesized and scrutinized for their inactivation of HNE [81]. Compounds have been modified by introducing halogen residues and different functional groups at varying positions resulting in derivatives with different activity and selectivity. 5-Chloropyrid-3-ylcoumarins proved to be highly active and specific for HNE. Due to the fact that these compounds are totally synthetic, they are only mentioned here, but no structures are given.

Chalcones may also serve as leads for inhibitors of HNE release. Thus, Hwang et al. synthesized different chalcone derivatives [82]. 3′-Isopropoxychalcone (structure see Fig. **10**) displayed the highest activity and prevented HNE release at a concentration of 3 μM to 30%. Activity was again related to inhibition of cellular cAMP levels through inhibition of phosphodiesterase.

These examples show the high potential of natural compounds as lead structures. Lead optimization tools such as QSAR studies or structure-based drug design can accelerate the discovery of promising new HNE inhibitors. Thus, Verma and Hansch published a QSAR study with caffeic acid derivatives [83]. Based on their 12 biological QSAR studies including those with HNE from Melzig et al. [35] they predict that the different activities of caffeic acid and its derivatives are mainly dependent on either their hydrophobicity or molar refractivity, with a bilinear correlation being the most important. This knowledge can be used for a better strategy to develop potent HNE inhibitors.

Another approach has been published by Steinbrecher et al., [84] which is suitable as a second step after virtual screening. When likely binding compounds are identified calculation of binding free energies may lead to new inhibitors with improved binding affinities as it has already been demonstrated with bornyl (3,4,5-trihydroxy)cinnamate (Steinbrecher et al manuscript in preparation).

Conclusion

Up to now reviews on HNE have been mainly focused on synthetic inhibitors [12], [33], [34]. Here for the first time natural compounds which are described to inhibit the enzyme or its release from neutrophils are reviewed. So far clinical studies have only been conducted with recombinant endogenous or synthetic elastase inhibitors [34], but not with natural or semi-synthetic compounds. One may question the significance of these natural compounds, because up to now none of them have been proven as highly active. However, it has to be kept in mind that the development of most synthetic inhibitors has been abruptly ended during clinical evaluation due to toxicity and side effects. The severity of diseases such as α1-AT deficiency, the worldwide increased morbidity of COPD [11], and especially the new findings on HNE as a key regulator of cell signalling during inflammation [4], [85], are reasons enough to continue evaluating the therapeutic potential of elastase inhibitors. Moreover, involvement of HNE in wound healing [4], in dermatological diseases such as psoriasis [16] as well as in wrinkle formation [2] open new fields in application of HNE inhibitors. Natural compounds, especially those which exert their inhibitory activity at low concentrations in a specific manner, should also be appreciated. Nevertheless, defined standardized plant extracts may also exibit beneficial effects, as very recently shown with an extract from *Zingiber officinale* . This extract significantly prevented wrinkle formation induced by chronic UV-B irradiation after topical application presumably due to its inhibition on fibroblast-derived elastase [86].

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