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Sesquiterpene Lactones as Inhibitors of Human Neutrophil Elastase

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Abstract—Human neutrophil elastase (HNE) is a serine protease that has been implicated in the abnormal turnover of connective tissue proteins and has been described as an important pathogenic factor in several inflammatory diseases such as rheumatoid arthritis or cystic fibrosis. Here we investigated 17 sesquiterpene lactones (SLs) for their ability to inhibit human neutrophil elastase in an in vitro assay. Podachaenin was the most active compound with an IC_{50} value of 7 μM. SLs do not covalently bind to the amino acids of the catalytic triad, thus differing from other 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 using the X-ray structure of HNE and the program FlexX revealed structural elements which are a prerequisite for their inhibitory activity.

Introduction

Preparations from many medicinal plants of the Asteraceae family, such as *Arnica montana* or *Tanacetum parthenium* are used for the treatment of inflammatory diseases.[1] The secondary plant metabolites that mediate these pharmacological effects are often sesquiterpene lactones (SLs). Several studies have shown that these compounds modulate many inflammatory processes, for example oxidative phosphorylation, platelet aggregation, histamine and serotonin release.[2,3] Recently, we have demonstrated that several SLs inhibit the transcription factors NF-κB and NF-AT.[4,5–7] These proteins promote the expression of a variety of target genes in response to inflammation, viral and bacterial infections and other stressful situations.[8,9] Consequently, downstream events, such as release of cytokines IL-1β, IL-6 or TNF-α production and lymphocyte proliferation are also inhibited by SLs.[10] These inhibitory activities are mediated chemically by α,β-unsaturated carbonyl structures which react with nucleophiles, especially cysteine sulfhydryl groups, by a Michael-type addition.[11,12]

Human neutrophil elastase (HNE) is a serine protease found in the azurophilic granules of neutrophils. This enzyme is a strongly basic glycoprotein with a specificity for small hydrophobic amino acids. During proteolysis the side chain of the amino acid is located in the S1 specificity pocket. Its backbone carbonyl is located in the 'oxyanion hole' and forms hydrogen bonds with the NH of Gly193 and Ser195 thus stabilizing the charged transition state. The catalytic triad is conserved in all serine proteases and consists of Ser195, His57 and Asp102.[13] The primary role of the intracellular HNE appears to be the proteolysis of foreign proteins during phagocytosis by neutrophils, whereas the main target for the extracellular elastase, which is secreted by neutrophils in the inflamed tissue, is the important connective tissue protein elastin. Elastin has the unique property of elastic recoil, is widely distributed in vertebrate tissue, particularly in lungs, blood vessels, skin and ligaments. HNE has a broad substrate specificity and may therefore attack a number of host proteins including elastin, proteoglycans, immunoglobulines, collagen and fibronectin outside the neutrophils. Under

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normal physiological conditions endogenous inhibitors like α1-antitrypsin protect healthy tissue from damage by elastase. However, intense neutrophil infiltration results in an impaired balance between the enzyme and its natural inhibitors. HNE can then cause abnormal degradation of healthy tissue resulting in the development of diseases such as rheumatoid arthritis, pulmonary emphysema, adult respiratory distress syndrome, cystic fibrosis or delayed wound healing.[14]

Here we investigated 17 SLs whether they also inhibit human neutrophil elastase (HNE) (for structures see Fig. 1, for name and origin see Table 1). This serine protease was shown to be inactivated by acylating agents with a lactone structure or by alkylating agents.[15] The SLs represent all major skeletal classes, including eudesmanolides, guaianolides, pseudoguaianolides and germacranolides. They differ from each other both in their molecular geometry and in the number of alkylating structure elements. Subsequently, the ligand docking program FlexX[16] was used in combination with the X-ray structure of 1HNE[17] to gain insights into the structure–activity relationship of the tested compounds from the predicted ligand–protein complexes.

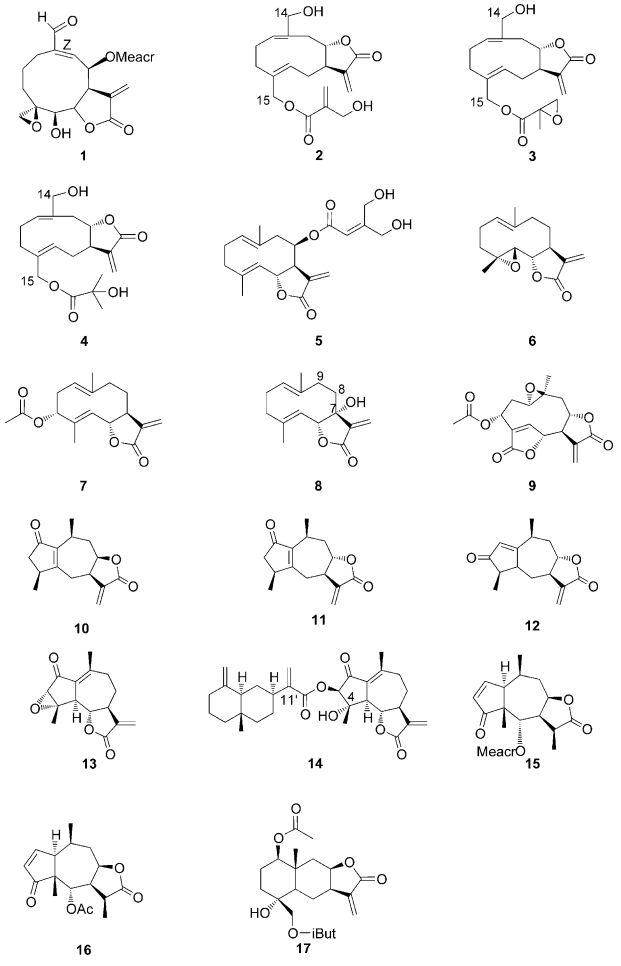

Figure 1. Structures of the investigated sesquiterpene lactones (Meacr = methacryloyl-, *i*But = isobutyryl-).

Results and Discussion

Elastase inhibition assay

In order to investigate whether SLs **1**–**17** influence the activity of HNE an in vitro assay was carried out with the isolated enzyme. All SLs possessed a lactone moiety, but they showed a different behaviour towards the enzyme resulting in different inhibitory concentrations necessary for a 50% inhibition. However, most of the SLs required a high concentration for the 50% inhibition. Therefore, HNE is probably not a preferable direct target for SLs.

According to their inhibitory activity SLs can be divided into two groups as shown in Table 2. Only the costic guaianyl ester podachaenin **14** exhibited a low IC_{50} value of 7 μM. Three SLs (**2**, **4**, **8**) possessed a moderate inhibitory activity with IC_{50} values being in the range of 15–33 μM. These four SLs are placed in group I. Compounds of group II inhibited either HNE at high concentrations or were inactive. These results are opposite to those which have been previously obtained in the NF-κB EMSA and the cytokine ELISA.[6,10] In these studies the inhibitory activity correlated with the number of α,β-unsaturated carbonyl structures, such as an α-methylene-γ-lactone or an α,β-unsaturated cyclopentenone. SLs which possessed in addition to an α-methylene-γ-lactone group at least one further α,β-unsaturated carbonyl group were much more active compared to those SLs with only one of these reactive structural elements. From the results with HNE it can be deduced that SLs must have an α-methylene-γ-butyrolactone group, because 11α,13-dihydrohelenalin derivatives **15** and **16** were less or non active. However, further features, such as hydroxy groups seem to be necessary for a strong activity against HNE (see compounds **2**, **4** and **8**).

Numerous studies have shown that the α-methylene-γ-butyrolactone group covalently binds in a Michael type reaction to nucleophiles, especially cysteine sulfhydryl groups.[11] Moreover, it is reported that most of the elastase inhibitors with a lactone structural element react in a covalent way with the amino acids in the active site of HNE.[14] Therefore, further experiments were performed with the most active SL **14** to differentiate between an irreversible mode of inhibition, for example acylation or alkylation of HNE by the inhibitor, and a reversible inhibition of the enzyme. Cysteine was used, because this amino acid is able to restore the elastase activity in the presence of an inhibitor which is non-covalently bound to the active site.

The inhibitory activity was totally abolished after subsequent addition of cysteine to the mixture of HNE and **14**. This can be due to the fact that cysteine reacts in a Michael-type addition with the activated exomethylene groups of **14**. Thereby the affinity of the cysteine adduct of **14** towards the active site of HNE is decreased. This reaction should only be observed if SL **14** is not covalently bound to an amino acid of the catalytic triad of HNE. For comparison we performed the same experiment with the irreversible inhibitor 2-methyl-4H-3,1-

Table 1. Investigated sesquiterpene lactones and their origin

SL No.	Name	Origin/reference
Germacranolides		
1	4β,15-Epoxy-miller-9*Z*-enolide	*Milleria quinqueflora*[18]
2	15-(3′-Hydroxy)-methacryloyloxy-micrantholide	*Mikania cordifolia*[19]
3	15-(2′,3′-Epoxy)-isobutyryloxy-micrantholide	*M. cordifolia*[19]
4	15-(2′-Hydroxy)-isobutyryloxy-micrantholide	*M. cordifolia*[19]
5	Eupatoriopikrin	*Eupatorium cannabinum*[a]
6	Parthenolide	Aldrich-Chemicals
7	3-Acetoxy-costunolide	*Podachaenium eminens*[20]
8	7-Hydroxy-costunolide	*P. eminens*[20]
9	Scandenolide	*Mikania micrantha*[d]
Guaianolides		
10	2-oxo-Guai-1(5)-en-12,8α-olide	*Decachaeta thieleana*[b]
11	2-oxo-Guai-1(5)-en-12,8β-olide	*D. thieleana*[b]
12	Thieleanin	*D. thieleana*[b]
13	Eminensin	*P. eminens*[20]
14	Podachaenin	*P. eminens*[20]
Pseudoguaianolides		
15	11α,13-Dihydrohelenalin- methacrylate	*Arnica montana*[c]
16	11α,13-Dihydrohelenalin- acetate	*A. montana*[c]
Eudesmanolide		
17	1β-Acetoxy-4α-hydroxy-15-isobutyryloxy-eudesma-11(13)-en-12,8β-olide	*M. cordifolia*[19]

[a]Generous gift from Prof. H. Becker, Saarbrücken.
[b]R. Murillo, V. Castro, A. X. Garcia-Piñeres, C. A. Klaas, I. Merfort, unpublished.
[c]Isolation procedure described in ref 21.
[d]Isolation: current thesis B. Siedle, identification in ref 22.

Table 2. Investigated SLs, their IC_{50} values in the elastase assay and the results of the ligand docking calculations

SL	IC_{50} (μM)	ΔG (kJ/mol)[a]	Oxyanion hole[b]	Side chains of His57 (○) or Ser195 (◇)[c]	Amino acids close to the active site[d]
	Group I (IC_{50} < 50 μM)				
14	7	−39	++	−	Phe41
2	15	−30	++	◇	Val216
8	29	−34	++	○	Asp194
4	33	−37	++	−[e]	Val216
	Group II (IC_{50} > 50 μM)				
7	70	−29	−	−	−
1	82	−33	−	−	Val216
9	91	−35	+	−	Val216
12	93	−32	++	−	−
3	99	−30	−	−	Val216
15	102	−34	−		Val216
13	110	−42	++	−	Val216
5	144	−29	+	◇	Phe41
6	> 100	−35	++	−	−
10	> 200	−48	++	−	Asp194, Val216
11	> 200	−30	++	−	−
16	> 200	−30	−	−	−
17	> 200	−39	++	○	−
	Control				
Caffeic acid	93		++		

[a]Calculated binding free energy for the highest ranked docking solution.
[b]Best docking with H-bonds to the oxyanion hole (backbone-NH of Ser195 and Gly193), ++ interaction with both NH-groups, + only interaction with Gly193NH, −no interaction with the oxyanion hole.
[c]Best docking with H-bonds to the catalytic active side chains of His57 or Ser195.
[d]Best docking with H-bonds to amino acids close to the active site.
[e]Best docking with H-bonds to the backbone carbonyl of His57.

benzoxazin-4-one.[23] In contrast to the SL **14** the proteolytic activity of elastase was not regenerated after addition of cysteine to the test mixture. From this experiment, it can be assumed that the exocyclic methylene group of the lactone moiety of SL **14** is not involved in the inhibitory activity, for example by covalent binding to a nucleophilic residue of HNE. Most probably this behaviour can be applied to the other SLs in this study, too. Therefore, concerning HNE SLs differ in their mode of action compared to most targets in which the activities are mediated chemically by the α-methylene-γ-lactone structural element.[11]

Ligand docking calculations

Selection of X-ray structure and scoring function. To explain the different activities of the SLs we performed docking calculations using the X-ray structure of 1HNE[17] and the program FlexX[16] Version 1.10.1. We chose the X-ray structure of 1HNE with the covalently bound inhibitor MeO-Suc-Ala-Ala-Pro-Val-CH_2Cl for the reasons that it had the highest resolution from all available elastase structures in the PDB-database (1.8 Å). It is preferable to use a structure complexed to an inhibitor because of the higher risk of unduly large structural changes owing to the unbound state in uncomplexed protein structures.[24] In the FlexX Version 1.10.1, the possibility to vary the scoring function is implemented. We chose the new scoring function ScreenScore,[25] which is a combination of the original FlexX scoring function[26] that performs best dockings for those target–ligand combinations that form a significant number of hydrogen bonds and the PLP scoring function[27] that is less dependent on hydrogen bonds and pronounces general steric fit. ScreenScore balances the weight of undirected lipophilic interactions and hydrogen bonds and fit well to our protein that has a rather lipophilic specificity pocket, and the active site with the oxyanion hole where the substrate is fixed by directed hydrogen bonds.

Ligand binding calculations. In contrast to cinnamic acid derivatives[28] it is not possible to explain the activity of the SLs with a uniform molecular inhibition mechanism from the docking experiments. However, as with the cinnamic acid derivatives the calculated binding free energies do not significantly correlate with the experimentally determined inhibitory concentrations. Regarding the ligand docking calculations for the SLs in group I, no general structure–activity relationship can be established, but explanations for the inhibitory activity can be given within structurally related SLs.

All three germacranolides isolated from *Mikania cordifolia* possess a melampolide skeleton with a hydroxy group at C-14, but they differ in the acidic side chain bound to C-15-OH. Interestingly, only **2** and **4** show a remarkable activity (IC_{50} 15 resp. 33 μM) whereas **3** is more or less inactive (IC_{50} 99 μM). This can be explained by the fact that **2** and **4**, but not **3** have an additional hydroxy group in the acidic side chain—what enables a double anchoring of the molecule in the active side (Fig. 2). The side-chain OH of **2** performs a hydrogen bond to the OH of the catalytically active Ser195 and to NH of Gly193, which is part of the oxyanion hole. The OH at C-14 can build a hydrogen bond to NH of Val216, which is an important amino acid of the S1 substrate specificity pocket. This anchoring probably prevents the natural substrate to bind to the catalytic triad. The best docking solution of **4** shows a 3-fold anchoring: the side-chain OH exhibits a hydrogen bond to the backbone carbonyl of the catalytically active His57, the lactone carbonyl is located in the oxyanion hole and interactions with Val216 NH are calculated for the C-14-OH group. In the docking experiments with compound

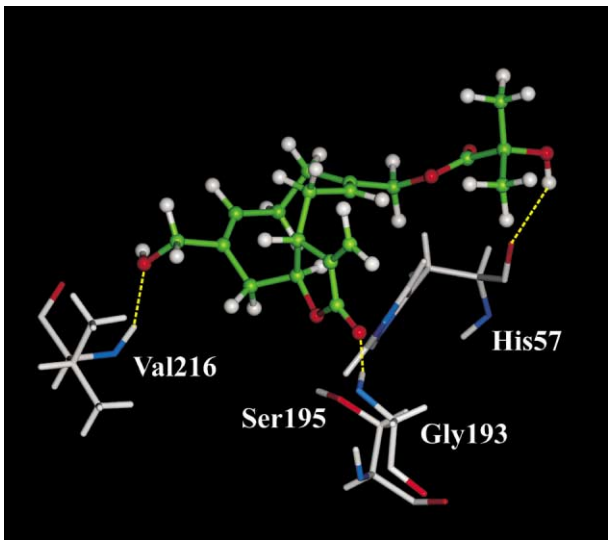

Figure 2. Highest ranked docking solution of SL **4** with the ligand bound to the active site. The dashed yellow lines indicate the hydrogen bonds between the ligand and HNE. Colour legend: carbon atoms of the protein: grey; carbon atoms of the ligand: green; oxygen: red; nitrogens: blue; hydrogens: white; sulfur: yellow.

3 no interaction is observed with the amino acids of the catalytic triad, corresponding to the high IC_{50} value. Despite of the few SLs of this structure type it can be assumed that the occurrence of two hydroxy groups, one in the acidic side chain and one at C-14, may be essential for the inhibitory activity against elastase.

Further ligand docking calculations were performed with 7-hydroxy-costunolide (**8**) and compared with those from SLs **6** and **7** to explain the relatively low IC_{50} value (33 μM) of **8** and the much more higher one of **7** (70 μM) as well as the nonactivity of **6** (>100 μM). The most conspicuous difference between these three SLs is the freely accessible hydroxy group at C-7 of **8**, which contributes to the double anchoring of the molecule in the binding pocket (Fig. 3). In detail: the carbonyl of the lactone ring is placed in the oxyanion hole, the hydroxy group forms a hydrogen bond to the Nε of the imidazole ring of His57 and the methyl group at C-14 contributes to the binding by hydrophobic interactions with the aromatic ring of Phe192. For parthenolide (**6**) the lactone carbonyl is predicted to be located in the oxyanion hole but there is no additional hydrogen bond donor structure to stabilize binding to the enzyme. For 3-acetoxy-costunolide (**7**) no placement is calculated in the near surroundings of the catalytic triad. The difference in activity towards the elastase shown in the experiments cannot be explained by the docking calculations and requires further studies. Thus, for this group of SLs a hydroxy function nearby the lactone carbonyl seems to be a prerequisite for a good interaction within the active site. To study theoretically the possible influence of the hydroxy position we undertook binding calculations using a SL in which the hydroxy group moved either to position 8 or 9. For the hypothetic structure with the hydroxy group in the 8α position the calculated docking solutions were similar to those for **7** with the

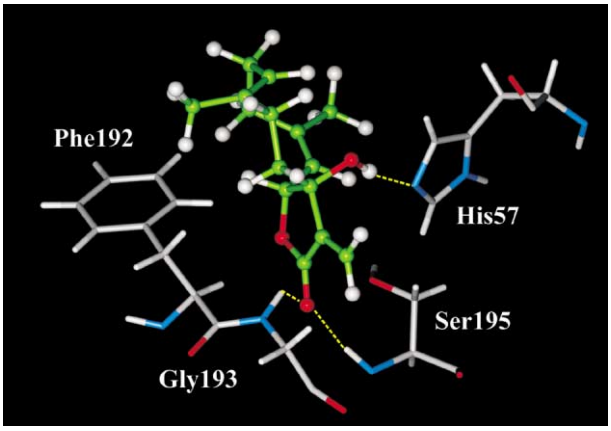

Figure 3. Highest ranked docking solution of 7-hydroxy costunolide (**8**) with the ligand bound to the active site. Colour legend see Figure 2.

lactone ring located in the oxyanion hole but no interactions between the side chain of His57 and the hydroxy group at C-8. No interactions with the oxyanion hole but a hydrogen bond between Ser195 OH and C-8 OH were calculated for the structure with the hydroxy group in the 8β position. Interactions to the active site could also be calculated for the structure with the hydroxy group in the 9β position, supporting the proposal that the orientation of the hydroxy group with regard to the lactone ring might be important for the interaction with the enzyme.

The most active SL podachaenin (**14**) (IC_{50} 7 μM) has a special position among all tested substances, because it is the only SL esterified with a relatively apolar cyclic acidic component. Docking calculations revealed that this structure is able to fill a major part of the furrow in which the peptidic substrate is normally bound in contrast to the smaller SLs **2**, **4** and **8** (Fig. 4). The carbonyl group of the acid component is fixed in the oxyanion hole, the free hydroxy group at C-4 exhibits a hydrogen bond to Gly193 NH and the methyl groups of the decalin part interact with the aromatic ring of Phe192. Moreover, the methylene group at C-11′ partly fills the S1 specificity pocket. Here again the hydroxy function nearby a carbonyl function seems to be an important feature for the inhibitory activity.

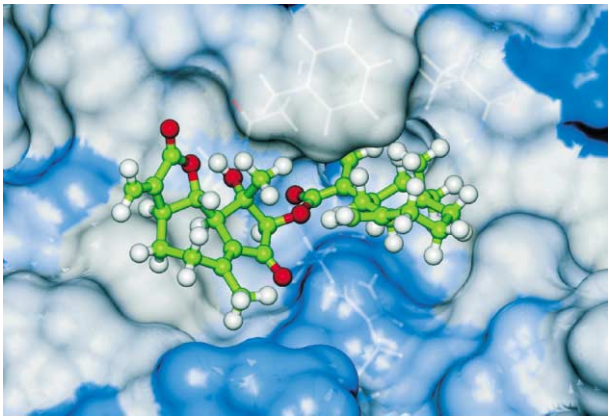

Figure 4. Highest ranked docking solution of podachaenin (**14**) with the ligand bound to the active site of HNE. The surface of the protein is coloured according to its hydrophobicity. Hydrophobic regions are coloured in white, polar regions in blue.

However, metabolism such as hydrolysis at pH 7.5 has to be taken into account under experimental conditions. Therefore we performed binding calculations for the hydrolyzation products, a decalin residue substituted with an acrylic acid and a SL with two vicinal hydroxy groups. This SL allows a stable placement in the active site with interactions of one hydroxy group to NH of Gly193 and to the hydroxy group of Ser195 and another to the carbonyl of Phe192. To confirm this hypothesis we investigated the stability of SL **14** at a pH of 7.5. However, GC and DC analysis revealed that the ester binding is stable under these conditions and hydrolysis only occurs to a low degree. Therefore, the existence of a freely accessible hydroxy group in a reasonable distance to a carbonyl function may be important for a favourable placement in the active side also in this case.

Conclusions

- SLs which inhibit HNE differ in their mode of action compared to other elastase inhibitors with a lactone structural element described so far. These compounds covalently bind to the amino acids of the catalytic triad whereas this type of binding is unlikely for the investigated SLs. In contrast to other biological activities of SLs inhibition of HNE is not mediated by α,β-unsaturated carbonyl functions.
- Ligand binding calculations revealed 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.
- Regarding the high concentrations which cause a 50% inhibition, HNE is not a direct target for most of the investigated SLs. However, it has to be elucidated whether SLs can indirectly inhibit HNE by influencing the release of cytokines up-regulating HNE.[29]

Experimental

Test compounds

18 SLs were isolated from different Asteraceae species as listed in Table 1.[18–20,30] 10 mM stock solutions were prepared in DMSO and then diluted with assay buffer.

Chemicals

Neutrophil elastase from human leukocytes (ICN, E.C. 3.4.21.37) was supplied by Fluka (Germany), the enzyme substrate MeO-Suc-Ala-Ala-Pro-Val-pNA and the soybean trypsin inhibitor were purchased from Sigma (Germany). Trichloroacetic acid, cysteine and 2-methyl-4H-3,1-benzoxazin-4-one were purchased from Sigma (Germany).

Elastase assay

The determination of the neutrophil elastase activity was performed according to Stein[31] and Löser et al.:[32] Briefly, 250 μL substrate solution (700 μM MeO-Suc-Ala-Ala-Pro-Val-pNA in Tris–HCl buffer, pH 7.5) were mixed with 100 μL test solution (test substances solubilized in Tris–HCl buffer, pH 7.5) and vortexed. After the addition of 250 μL enzyme solution (approximately 0.5 mU) the samples were incubated for 1 h at 37 °C. The reaction was stopped by addition of 500 μL soybean trypsin inhibitor solution (2 mg/mL Tris–HCl buffer, pH 7.5) and placed in an ice bath. After vortexing the absorbance of the free *p*-nitroaniline was measured at 405 nm.

Elastase assay with cysteine

The enzyme was incubated with the inhibitor (12,5 μM of **14** and 50 μM of 2-methyl-4H-3,1-benzoxazin-4-one) in assay buffer for 30 min. After that time, 10 μL of a cysteine solution (50 mM) and 250 μL of the substrate solution (700 μM MeO-Suc-Ala-Ala-Pro-Val-pNA) was added. The reaction was stopped after 1 h and the absorbance was measured as described before. Additionally, the enzyme was only incubated with the inhibitor. The enzymatic reactivation was determined by comparing the enzyme activity from both experiments. A solution containing no inhibitor was used for control.

Statistical analysis

All assays were performed at least three times with duplicate samples. Inhibition rates were calculated in percent to control without inhibitor. IC_{50} values were determined from dose–effect curves by linear regression. All IC_{50} values were in between the 95% confidence interval.

Ligand docking calculations

The X-ray structure of HNE from Navia et al.[17] (PDB code: 1HNE) was used in our calculations after deletion of the irreversible inhibitor MeO-Suc-Ala-Ala-Pro-Val-CH_2Cl from the PDB file. Its binding site was determined with the molecular modeling package WhatIf.[33] All amino acids were included with at least one atom lying within a 6.5 Å distance from the inhibitor. Polar hydrogen atoms were placed with the HB2NET command of WhatIf, optimizing the hydrogen bond network of the complete protein.[34] Crystallographically determined water molecules were omitted for the docking calculations as they were not found to be located in the active site region.

Low-energy conformations of the SLs were generated using the conformational search option of the molecular modeling package Hyperchem® (v. 6.02). Energy minimizations were performed with Hyperchem's force field MM+ using the Polak-Ribière minimization algorithm. Starting structures were created with Hyperchem and initially minimized to an RMS gradient <0.01 kcal mol^{-1} $Å^{-1}$. All rotatable cyclic bonds were included as variable torsions and allowed to be changed simultaneously. The search was performed applying a usage directed search method and standard settings for duplication tests. A search run was terminated after energy minimization of 2500 unique starting geometries. The conformers with the lowest energy were used as input files for the ligand docking calculations. Acyl side chains of the respective SLs were not included in the conformational search. They were added manually to the most favourable SL conformers and the resulting models were energy minimized to an RMS gradient as above.

Docking calculations were carried out on a PC with SuSE Linux 7.3® as operating system with the program FlexX 1.10.1 (GMD, Germany),[16] which is designed for the docking of small to medium sized organic molecules into protein binding sites. During the docking calculations the ligand is considered as flexible, that is rotations are possible around all acyclic single bonds and up to seven-membered rings are considered as flexible. The ligand structure is divided into components by cutting at each acyclic non-terminal single bond. Then a maximum of three components are combined to a fragment. A base fragment is selected and the binding site, which is considered as rigid, is searched for optimal interaction points. After this 'base placement' operation the ligand is incrementally built up. Directed chemical interactions (predominantly hydrogen bonds and salt bridges) as well as hydrophobic interactions of the ligand with the receptor were used for the calculation of ligand placements applying an incremental construction algorithm. The interaction geometries were deduced from the analysis of crystallographic data. The predicted protein-ligand complexes were optimized and ranked according to the empirical scoring function ScreenScore,[25] which estimates the binding free energy, ΔG^0, of the ligand receptor complex. The best placement refers to the ligand receptor complex of the lowest binding free energy. Lower ranked docking solutions were also considered and confirmed the results of the best one.

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