Institute of Pharmacy[1], Free University Berlin, Germany, and Chemistry Department[2], Pomona College, Claremont, USA

Inhibition of neutrophil elastase and thrombin activity by caffeic acid esters

M. F. Melzig[1], G. D. Tran[1], K. Henke[1], C. D. Selassie[2], R. P. Verma[2]

Dedicated to Prof. Dr. Eberhard Teuscher on the occasion of his 70th birthday

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Prof. Dr. Matthias F. Melzig, Institute of Pharmacy, Free University Berlin, Königin-Luise-Str. 2 + 4, D-14195 Berlin, Germany
melzig@zedat.fu-berlin.de

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Natural and synthetic caffeic acid esters were assayed for their enzymatic activity versus neutrophil elastase (EC 3.4.21.37) and thrombin (EC 3.4.21.5). Lipophilic caffeic acid esters inhibited neutrophil elastase activity and the inhibition rate was enhanced with increasing length of the aliphatic chain of the alcohol component. The geometry of the chain seems to be more important than the number of carbon atoms. The most inhibitory compound was *n*-octylcaffeic acid ester with an IC_{50} value of 1.0 μM. Thrombin activity was only weakly inhibited by the caffeic acid esters thus demonstrating a specificity for neutrophil elastase. Because of its critical role in inflammatory processes, inhibition of neutrophil elastase by caffeic acid esters might be of importance in the treatment of inflammation.

1. Introduction

Neutrophil elastase (leukocyte elastase, EC 3.4.21.37) belongs to the chymotrypsin family of serine proteinases (S1), and is formed of a single peptide chain of 218 amino acid residues and four disulfide bridges (Powers and Harper 1986). Human neutrophil elastase was first identified in 1968; the term elastase describes an enzyme capable of the proteolytic release of soluble peptides from insoluble elastin (Janoff and Scherer 1968).

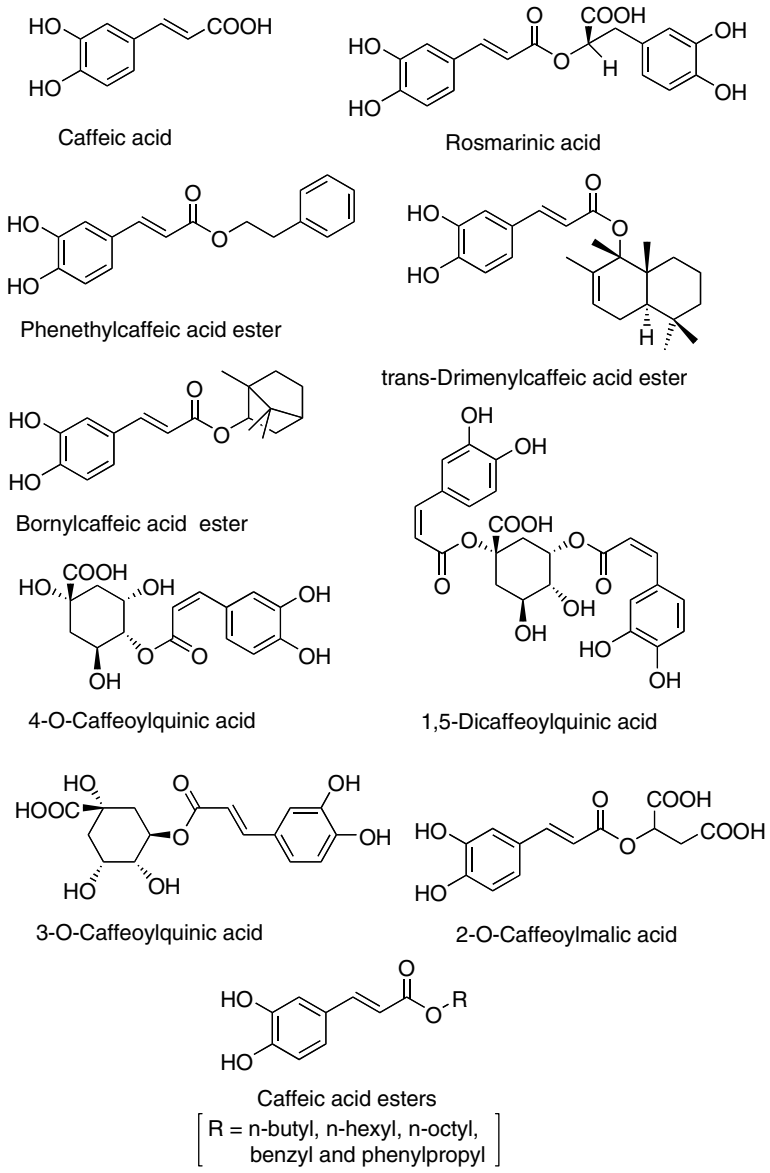
The major physiological function of neutrophil elastase is probably to digest bacteria and immune complexes phagocytosed by the polymorphonuclear leukocyte. Human neutrophil elastase has been implicated in the pathogenesis of different inflammatory diseases, like hereditary emphysema, cystic fibrosis, chronic obstructive pulmonary disease, the adult respiratory distress syndrome (ARDS), rheumatoid arthritis and infectious diseases (Bieth 1998).

A broad screening of natural compounds as elastase inhibitors has recently been conducted and revealed the inhibitory potential of caffeic acid esters from plants (Melzig et al. 2001). In view of these results we synthesized new caffeic acid esters and investigated their inhibitory potential against neutrophil elastase and thrombin (EC 3.4.21.5) another serine protease, in order to assess the specificity of inhibition.

2. Investigations, results and discussion

Neutrophil elastase is released from neutrophils that migrate to sites of infection or other damaged tissues during the early stages of inflammation. It accumulates due to ineffective phagocytosis and apoptosis and is consequently a useful marker of inflammation (Liou and Campbell 1995). Under physiological conditions neutrophil elastase activity is required after acute connective tissue injury, such as wounds, in the initial stage of tissue repair (Murphy and Reynolds 1993). In a chronic inflammatory process the role of neutrophil elastase activity is changed to a detrimental pathological one when the protease/antiprotease balance is overwhelmed and subsequently promotes inflammation and inhibits the process of healing. For these chronic inflammatory processes inhibitors of neutrophil elastase represent a crucial strategy in the treatment of pulmonary disease.

Initial investigations in inhibitory studies of neutrophil elastase by phenolic compounds from medicinal plants revealed the following: flavonoids with the quercetin aglycone and different lipophilic caffeic acid esters used for the treatment of inflammatory diseases were effective inhibitors of neutrophil elastase. These results led us to hypothesize that esterification with a lipophilic alkoxy moiety could increase the inhibitory activity of caffeic acid esters (Melzig et al. 2001). To validate this assumption we systematically investigated caffeic acid esters with increasing number of C-atoms of the alcohol component. The results are shown in Table 1. It is clear that the inhibition of neutrophil elastase is enhanced with increasing length of the aliphatic chain of the alcohol moiety. The geometry of the chain seems to be more important than the number of C-atoms because the isopropyl-, tert-butyl-, benzyl-, phenylethyl-, and phenylpropyl caffeic acid esters show a lower IC_{50} compared to the unbranched aliphatic esters. Stearyl- and palmityl caffeic acid esters were synthesized, but these compounds were almost insoluble in the assay buffer and did not reduce neutrophil elastase activity in concentrations of 50 μM. Their insolubility precluded further enzymatic analysis at higher concentrations. Comparison between the synthetic caffeic acid esters and the natural products indicates that



the catecholic functional groups are of critical importance in the inhibitory process. A quantitative structure-activity relationship (QSAR) analysis was conducted to shed some light on the physicochemical attributes that contribute to inhibitory potency.

Combining the results in Table 1 with results previously obtained versus naturally occurring caffeic acid esters (Melzig et al. 2001), led to the formulation of Eq. (1) for the inhibition of human neutrophil elastase (Table 2).

$$\text{Log } 1/IC_{50} = 0.34(\pm 0.12)\,\text{Clog P} + 3.93(\pm 0.42) \quad (1)$$
$$n = 14\,, \quad r^2 = 0.752\,, \quad s = 0.527\,, \quad q^2 = 0.688$$

Exclusion of the benzyl ester of caffeic acid which has a standard deviation almost twice that of Eq. (1) leads to the formulation of Eq. (2).

$$\text{Log } 1/IC_{50} = 0.35(\pm 0.10)\,\text{Clog P} + 4.00(\pm 0.35) \quad (2)$$
$$n = 13\,, \quad r^2 = 0.840\,, \quad s = 0.428\,, \quad q^2 = 0.795$$

In these equations, IC_{50} represents the concentration of each compound that induces 50% inhibition of elastase activity. Log $1/IC_{50}$ is the dependent variable in this study and defines the biological parameter for these QSAR. Clog P is the calculated partition coefficient of each compound. QSAR 1 and 2 both suggest that inhibitory activity increases with an increase in overall hydrophobicity.

The coefficient with the hydrophobic term in Eq. (2) is 0.35. This low value suggests that the caffeic acid esters are partially desolvated on the surface of a hydrophobic binding site in human neutrophil elastase. Extensive QSAR studies of receptor-ligand interactions have suggested that coefficients between 0.4 and 0.6 are indicative of partial desolvation of a ligand on a receptor surface while values between 0.7 and 1.1 pertain to total desolvation of the ligand (Hansch and Klein 1986). Eq. (2) accounts for 84% of the variance (r^2) of the data. The cross-validated r^2 (q^2), which is relatively high (0.80) was obtained by using the leave-one-out procedure (Cramer et al. 1988). Another strategy involving Y-randomization was also used to validate QSAR model 2 (Tropsha et al. 2003). The biological data was randomly shuffled five times and the following statistical values were obtained: ($r^2 = 0.50$, $q^2 = 0.34$; $r^2 = 0.14$, $q^2 = -0.16$; $r^2 = 0.0$, $q^2 = -0.38$;

Table 1: Enzyme inhibition by caffeic acid esters

Substance	IC_{50} (μM) Neutrophil elastase	IC_{50} (μM) Thrombin
Phenethylcaffeic acid ester	37	85
Rosmarinic acid	7	82
Methylcaffeic acid ester	>400 (23% inhibition at 400 μM)	>2000 (17% inhibition at 2 mM)
Ethylcaffeic acid ester	>400 (31% inhibition at 400 μM)	>2000 (28% inhibition at 2 mM)
n-Butylcaffeic acid ester	8	519
n-Hexylcaffeic acid ester	5	>2000 (35% inhibition at 2 mM)
n-Octylcaffeic acid ester	1	>2000 (46% inhibition at 2 mM)
Isopropylcaffeic acid ester	>400 (40% inhibition at 400 μM)	>2000 (44% inhibition at 2 mM)
Benzylcaffeic acid ester	145	500
Phenylpropylcaffeic acid ester	22	340
tert-Butylcaffeic acid ester	>400 (38% inhibition at 400 μM)	>2000 (45% inhibition at 2 mM)
n-Hexadecylcaffeic acid ester	n.d.	n.d.
n-Octadecylcaffeic acid ester	n.d.	n.d.

n.d. not determinable

$r^2 = 0.15$, $q^2 = -0.16$; $r^2 = 0.01$, $q^2 = -0.44$). It is clear from the Y-randomization test that these low r^2 and low LOO q^2 values ensure the robustness of model 2.

The benzyl ester of caffeic acid is overpredicted by a factor of sixteen. This suggests that some other constraints such as steric factors might be operative. It is of interest to compare the behavior of aromatic containing alkoxy moieties in this data set (Table 3).

These results suggest that the aromatic ring is at a critical distance at the reactive site where it may encounter some steric hindrance from an amino acid residue that subsequently results in weaker binding at the active site. As the methylene bridge elongates as in compounds **3** and **14**, there is greater flexibility which may allow for enhanced maneuverability at this critical site; thus the mispredictions in activity are not as severe as in the case of the benzyl ester.

Methylcaffeic acid ester and ethylcaffeic esters were evaluated for their inhibitory potencies versus neutrophil elastase but showed minimal inhibition (<31%) at 400 μM. It can thus be assumed that their log $1/IC_{50}$ values would be <3.40 if high inhibitory concentrations were attainable. Based on QSAR 2, the predicted values of the methyl and ethyl analogues would be around 4.41 and 4.60, respectively. These two compounds would also be mispredicted by a factor of 15, which again suggests that the methyl and ethyl linkage are buttressing against a residue that is critical to binding. On the other hand, the butyl, hexyl and octyl analogues with their flexible methylene linkages are very well predicted.

QSAR 2 suggests that slightly more hydrophobic analogues could be potent inhibitors of elastase. However, the enhanced hydrophobicity of *n*-hexadecylcaffeate (Clog P = 9.14) and *n*-octadecylcaffeate (Clog P = 10.19) prevented their facile dissolution in the assay buffer and subsequent assessment. The *n*-octyl caffeate is one of the most potent inhibitors to date. It is 37 times more effective at inhibiting neutrophil elastase than CAPE. It is of interest to note that *n*-octylcaffeate is almost ten times as potent as CAPE at inhibiting cellular growth versus L1210 cells in culture and three times more cytotoxic than CAPE in MCF-cells (Etzenhouser et al. 2001).

N-Octylcaffeate might be an interesting candidate in further pharmacological tests for the treatment of chronic inflamed wounds on the surface of skin or mucosa. The

Table 2: Biological and physicochemical constants used to derive QSAR 1 and 2 for the inhibition of human leukocyte elastase by caffeic acid esters

Compd.		Log $1/IC_{50}$			Clog P
		Obsd.	Pred.[b]	Pred.[c]	
1	Caffeic acid[a]	4.03	4.26	4.34	0.97
2	Rosmarinic acid[a]	5.15	4.31	4.38	1.10
3	Phenethylcaffeic acid ester[a]	4.43	5.05	5.15	3.30
4	*Trans*-drimenyl caffeic acid ester[a]	6.70	6.30	6.43	6.98
5	Bornyl caffeic acid ester[a]	5.80	5.65	5.76	5.06
6	1,5-Dicaffeoyl quinic acid[a]	3.82	3.86	3.93	−0.20
7	4-*O*-Caffeoyl quinic acid[a]	3.32	3.46	3.51	−1.40
8	3-*O*-Caffeoyl quinic acid[a]	3.35	3.29	3.34	−1.88
9	2-*O*-Caffeoyl malic acid[a]	4.47	3.96	4.02	0.07
10	*n*-Butylcaffeic acid ester	5.10	4.88	4.97	2.79
11	*n*-Hexylcaffeic acid ester	5.30	5.24	5.34	3.85
12	*n*-Octylcaffeic acid ester	6.00	5.60	5.70	4.90
13	Benzylcaffeic acid ester[d]	3.84	4.94	5.03	2.97
14	Phenylpropylcaffeic acid ester	4.66	5.18	5.28	3.68

[a] according to Melzig et al. (2001)
[b] calculated using eq. (1)
[c] calculated using eq. (2)
[d] not used in the derivation of eq. (1)

Table 3: Overprediction of arylalkoxy esters of caffeic acid

Compd.	−OR′	Deviation[a]	Overprediction factor
13	$OCH_2C_6H_5$	−1.19	16
3	$OCH_2CH_2C_6H_5$ (CAPE)	−0.72	5
14	$OCH_2CH_2CH_2C_6H_5$	−0.62	4

[a] according to eq. (2)

inhibition of elastase activity is connected with a decrease of matrix protein degradation and results in the attenuation of the progression of the inflammatory process. The bioavailability of caffeic acid esters *in vivo* seems to be adequate, since these compounds are easily absorbed after oral administration (Azuma et al. 2000; Caccetta et al. 2000; Takenaka et al. 2000).

The caffeic acid esters show little or no inhibitory activity against the serine protease thrombin (Table 1), which demonstrates that the caffeic esters inhibit the neutrophil elastase in a rather specific manner. Thrombin activity is inhibited by the synthesized caffeic acid esters only at high concentrations (in the millimolar range) that subsequently lack pharmacological significance. An exception might be both natural compounds rosmarinic acid and phenylethylcaffeic acid ester (CAPE) with IC_{50} values around 80 μM. Such concentrations are present in plant extracts or propolis used for local treatment on mucosal tissues. The inhibition of thrombin activity prevents the activation of proteinase-activated receptors of subtype PAR1, 3 and 4 included in inflammation, immune response and digestion disorders (Macfarlane et al. 2001). A synergistic activity due to inhibition of activity of neutrophil elastase and thrombin can be assumed.

3. Experimental

3.1. Materials

Caffeic acid phenethylester, rosmarinic acid, bovine thrombin and the elastase substrate MeO-Suc-Ala-Ala-Pro-Val-pNA was purchased from Sigma (Germany), while human neutrophil elastase was supplied by ICN. The thrombin substrate H-D-Phe-Homopro-Arg-p-NA diacetate was purchased from Bachem (Germany). All other chemicals were from Sigma (Germany).

3.2. Synthesis of caffeic acid esters

Melting points were determined on an electro thermal melting point apparatus (MEL-TEMP II with digital thermometer). ^{1}H NMR was recorded on a Bruker DPX 400 MHz NMR spectrometer with TMS as the internal standard; chemical shifts are given in δ (ppm). Thin-layer chromatography was performed on silica gel plates (silica gel IB-F Baker). Chemical elemental analysis was carried out by Desert Analytics (Tucson, AZ).

All the caffeic acid esters used in this paper were either commercially available or previously prepared compounds (Etzenhouser et al. 2001) except: caffeic acid hexadecyl ester (*n*-hexadecyl caffeate) and caffeic acid octadecyl ester (*n*-octadecyl caffeate), which were synthesized by the alkylation of the caffeic acid carboxylate salt as follows (Hashimoto et al. 1988).

Caffeic acid hexadecyl ester (*n*-hexadecyl caffeate): 1.2 ml of aqueous NaOH solution (2.8 g NaOH in 10 ml of H_2O) was added slowly to the solution of caffeic acid (1.0 g, 5.5 mmol) dissolved in 40 ml of *N,N*-dimethylformamide (DMF). The mixture was stirred for 2.5 h. A solution of 1-bromohexadecane (7.3 ml, 24 mmol) in DMF (10 ml) was added dropwise and the mixture was stirred at room temperature for 12 days. The reaction mixture was then poured into iced water (1.2 l) and extracted with diethyl ether (400 ml). The ether extract was washed successively with 1 N HCl (3 × 150 ml), H_2O (3 × 150 ml) and brine (2 × 75 ml). The extract was then dried over anhydrous $MgSO_4$, filtered and concentrated in a rotary evaporator, to yield an oily residue. The oily residue was subjected to column chromatography. Elution with chloroform-ethylacetate (8 : 2 v/v) gave a bright yellow colored compound that was identified as *n*-hexadecyl caffeate. Yield (pure): 1.06 g (47.17%), m.p. 106–107 °C. ^{1}H NMR (DMSO-d_6, ppm): δ = 0.85 (t, 3 H), 1.18–1.38 (m, 26 H), 1.61 (q, 2 H), 4.09 (t, 2 H), 6.25 (d, J = 16.2 Hz, 1 H), 6.75 (d, J = 7.98 Hz, 1 H), 6.99 (dd, J = 8.5, 2.0 Hz, 1 H), 7.04 (d, J = 2.0 Hz, 1 H), 7.45 (d, J = 15.6 Hz, 1 H), 9.35 (broad singlet, 2 H). Anal. Calcd ($C_{25}H_{40}O_4$): 74.22% C, 9.97% H; Found 74.30% C, 10.25% H. The structure of this compound has been further confirmed by comparing the spectral data with literature values (Nagaoka et al. 2002).

Caffeic acid octadecyl ester (*n*-octadecyl caffeate): The process used, was similar to that described for *n*-hexadecyl caffeate, except 1-bromooctadecane was used in place of 1-bromohexadecane. The crude product was subjected to column chromatography, elution with chloroform-ethyl acetate (8 : 2 v/v) and gave pure *n*-octadecyl caffeate. Yield (pure): 880 mg (36.62%), m.p. 109–110 °C. ^{1}H NMR (DMSO-d_6, ppm): δ = 0.85 (t, 3 H), 1.15–1.38 (m, 30 H), 1.60 (q, 2 H), 4.09 (t, 2 H), 6.26 (d, J = 16.1 Hz, 1 H), 6.75 (d, J = 7.7 Hz, 1 H), 7.0 (dd, J = 9.1, 2.0 Hz, 1 H), 7.05 (d, J = 2.0 Hz, 1 H), 7.46 (d, J = 15.4 Hz, 1 H), 9.4 (broad singlet, 2 H). Anal. Calcd ($C_{27}H_{44}O_4$): 74.96% C, 10.25% H; Found 75.01% C, 10.47% H.

All test compounds were dissolved in DMSO (10 mM stock solution) and then diluted with assay buffer. The influence of DMSO on enzyme activity was considered and normalized in controls.

3.3. Elastase assay

The determination of neutrophil elastase activity was performed with human leukocyte elastase (Löser et al. 2000). 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 was read at 405 nm. Inhibition rates were calculated in percent to controls without inhibitors. IC_{50} values were determined from dose-response-curves by linear regression.

3.4. Thrombin assay

The determination of thrombin activity was performed in a kinetic assay using bovine thrombin according to Dong et al. (1998) in 96-multiwell plates (Greiner). Briefly, 20 μL thrombin (1.31 NIH units/mL) in Tris buffer (50 μM + 0.1 M NaCl, pH 7.5), 50 μL of various concentrations of test compounds and 110 μL Tris buffer (pH 7.5) were preincubated for 5 min at 37 °C in each well. Then 20 μL substrate solution (2.475 mM H-D-Phe-Homopro-Arg-p-NA diacetate in Tris buffer, pH 7.5) were added and the increase of absorbance at 405 nm was measured over 30 min in a microplate autoreader (Tecan Spectra Fluor). The slope of each reaction (time versus optical density at 405 nm) was analyzed by linear regression and used for calculation of the inhibition rates. In each experiment two blind samples without test compounds were measured. IC_{50} values were determined by linear regression from dose-response-curves.

All assays were performed at least three times with triplicate samples.

3.5. QSAR Analysis

The QSAR analysis was carried out by using the C-QSAR program (BioByte Corp. Claremont, CA 91711, www.biobyte.com).

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