FOLIA HISTOCHEMICA ET CYTOBIOLOGICA
Vol. 45, No. 4, 2007
pp. 291-313

Review article

Cathepsin D inhibitors

Marek Gacko[1], Alina Minarowska[2], Alicja Karwowska[3], Łukasz Minarowski[4]

Departments of: [1]Vascular Surgery, [3]Instrumental Analysis, [4]Clinical Molecular Biology, Medical University of Bialystok, Poland;
[2]3rd Department of Children's' Diseases, Medical University of Bialystok, Poland

Abstract: Inhibitors of cathepsin D belong to chemical compounds that estrify carboxyl groups of the Asp33 and Asp231 residues of its catalytic site, penta-peptides containing statin, *i.e.* the amino acid similar in structure to the tetraedric indirect product, and polypeptides found in the spare organs of many plants and forming permanent noncovalent complexes with cathepsin. Cathepsin D activity is also inhibited by alpha2-macroglobulin and antibodies directed against this enzyme. Methods used to determine the activity and concentration of these inhibitors and their analytical, preparative and therapeutic applications are discussed.

Key words: Cathepsin D - Inhibitors - Aspatyl endopeptidase

Cathepsin D (EC 3.4.23.5) is an aspartyl endopeptidase, like cathepsin E, pepsin, gastrixin and rennin. Aspartyl endopeptidases are synthesized by malaria sporozoites, sheep liver fluke *Fasciola hepatica*, fungi Candida albicans and human immunodeficiency virus HIV [1-5]. Cathepsin D is synthesized in the rough endoplasmic reticulum (RER) in a form of preprocathepsin D, built up of 412 amino acid residues [6,7]. The RER is the site of glycosylation and formation of disulphide bridges in preprocathepsin D. Splitting off of the N-terminal 20 aminoacid prepeptide by signalase causes release of procathepsin D and its passage to the Golgi apparatus and primary lysosomes. In an acid environment of the primary lysosomes, intramolecular autocatalytic splitting off of the 44 amino acid propeptide and the formation of active enzyme occur [8-12]. The actions of cysteine proteases, aminopeptidases, carboxypeptidases and the autocatalytic process lead to the formation of a mature two-chain cathepsin D molecule, 12 + 34 kDa, [13,14]. These protease inhibitors inhibit maturation of cathepsin D [15], which can also be found on the cell surface due to fusion of lysosomes with the plasma membrane [16,17]. Cathepsin D may pass to cytosole, to the intracellular environment and to the blood.

Correspondence: A. Karwowska, Dept. of Instrumental Analysis, Medical University of Bialystok, Mickiewicza Str. 2b, 15-230 Białystok, Poland; tel.: (+4885) 7485600, e-mail: alicjakarwowska@yahoo.com

The catalytic site of cathepsin D consists of two aspartic acid residues: Asp33 and Asp231 [18], which together with water molecule cause hydrolysis of peptide binding. The ionized carboxyl group of the Asp33 residue activates the water molecule and facilitates proton detachment. The protonated carboxyl group of the Asp231 residue, in an acid environment, polarizes the carboxyl group of the substrate peptide binding, facilitates formation of indirect tetraedric product and increases its vulnerability to cleavage. The cleavage refers to the peptide binding situated within the polypeptide chain formed by carboxyl groups of hydrophobic amino acids [19].

Cathepsin D is involved in the process of degradation of exploited and denaturated cell proteins and those reaching the cell via endocytosis [20,21]. It also affects proteins indirectly through activation of procathepsin B [22,23], procathepsin L [24] prorenin [25] and inactivation of cystatins [26,27], stephins [26], and inhibitors of seryl protease type 1-2 [28], urokinase type 1-3 [28, 29], α1-antithrypsin [30], α1-antichymothrypsin [31] and calicrein [31]. Cathepsin D is also engaged upon selective proteolysis, which leads to the activation of prohormons and biologically active peptides and to the inactivation of their active forms [16,27,32-37]. Moreover, it takes part in the formation, development and regression of such pathological changes as inflammatory states, atherosclerosis, intravascular thrombi, apoptosis, mutagenesis, neoplastic proliferation or Alzheimer disease [38-53].

Cathepsin D activity is regulated on a few levels: through stimulation and inhibition of its biosynthesis, at the stage of post-translatory modifications in the Golgi apparatus, proenzyme activation and interlysosomal pH regulation [54-56]. This ensures that cathepsin D can operate at the proper time, site and intensity, and prevents uncontrolled protein degradation. The 20-kDa cytoplasmic protein, glycine ethyl ester, tripolyphosphates, phospholipids and ATP increase the activity of cathepsin D [57-59].

Cathepsin D degrades proteins at pH=3.5-5.5 [60]. Binding of this protease to a constant carrier shifts digestion towards neutral pH [61]. Vulnerability to degradation by cathepsin D depends also on the protein molecular structure. Haemoglobin and other denaturated proteins are more prone to the action of this protease than native proteins. However, complexification of haemoglobin with haptoglobin causes a reduction in the optimum pH from 3.5 to 2.8 and at the same time a 50% decrease in degradation rate [62].

Definition and division of cathepsin D inhibitors

The inhibitor is a compound showing chemical affinity toward an enzyme or bearing structural similarity to a substrate which binds to the catalytic site of an enzyme and forms an inactive enzyme-inhibitor complex. Chemical compounds reducing the enzyme activity via another mechanism are referred to as inactivators. Inactivators do not exhibit specificity for a particular enzyme or group of enzymes and include such protein-denaturating agents as heavy metal salts, alkaloid reagents, tannins, detergents, acids and bases, and elevated temperature. The inhibitors can be divided into competitive, noncompetitive and mixed, immediate and progressive, monovalent and polyvalent, synthetic and natural, endogenous and exogenous. The Lineweaver-Burk equations and plots are used to determine the competitive, noncompetitive and mixed types of inhibitors [63-66]. Based on the experimental data obtained for various inhibitor concentrations, the Michaelis constant and the maximum velocity are determined. The values obtained are placed in a plot showing the relationship of the reciprocal of the Michaelis constant and the reciprocal of the maximum velocity with the inhibitor concentration. Depending on the type of inhibitor, the curves may show either increased inclination or a shift of the point of intersection with the axis of ordinates. The competitive inhibitor shows structural similarity to a substrate with which it competes for an enzyme's catalytic site. It reduces substrate affinity for an enzyme and increases the Michaelis constant (1/Km). However, the maximum velocity, under substrate excess, remains stable (1/Vmax). This causes increased inclination of the

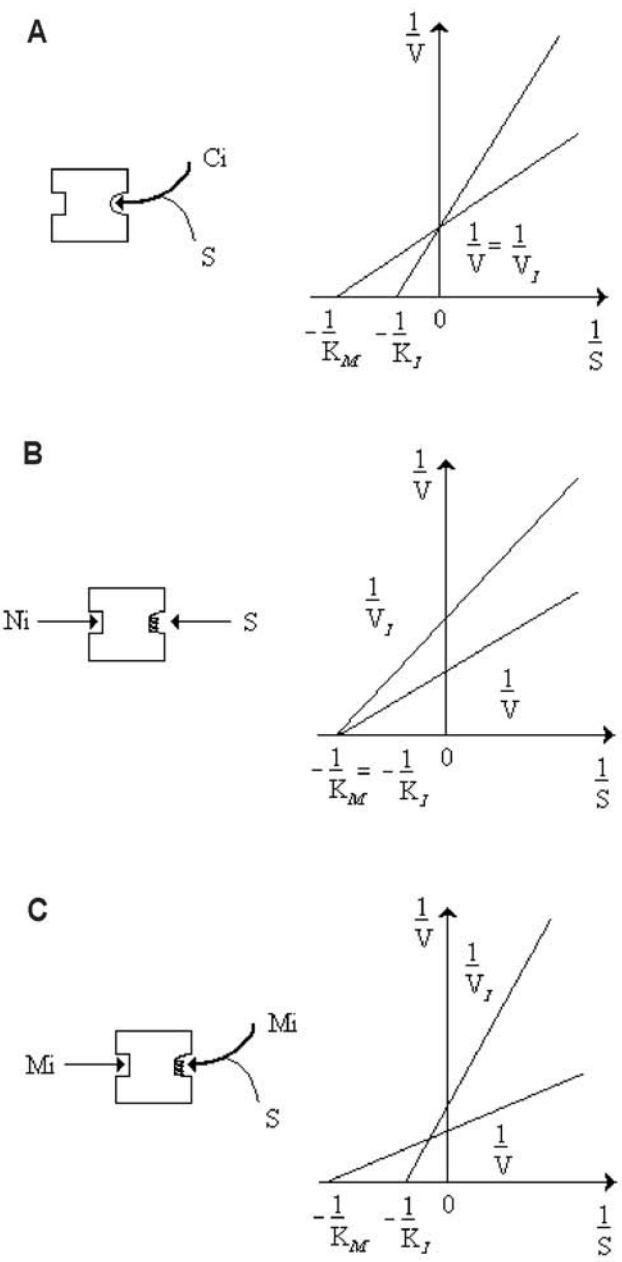


Fig. 1. Determination of the inhibition constant by the double-reciprocal plot method; **A** - competitive inhibition, **B** - noncompetitive inhibition, **C** - inhibition of the mixed type; Ci - competitive inhibitor, Ni - noncompetitive inhibitor, Mi - mixed type inhibitor [63].

Lineaweaver-Burk plot, at the same point of intersection with the axis of ordinates as when the inhibitor is absent (Fig. 1A). The noncompetitive inhibitor has the substrate structure and binds to the enzyme outside the catalytic site. Such an inhibitor does not alter the Michaelis constant (1/Km), but decreases the maximum reaction velocity (1/Vmax), which leads to increased inclination of the curve and to a shift of the point of intersection with the axis of ordinates (Fig. 1B). The mixed inhibitor, which is partly competitive

Table 1. Properties of different types of inhibitors: competitive, noncompetitive and mixed type.

Property	Inhibitor type		
	competitive	noncompetitive	mixed type
Structural similarity to substrate	similar	not similar	partly similar
Binding site	catalytic site	outside catalytic site	catalytic site and outside catalytic site
Reversibility through substrate surplus	reversible	not reversible	partly reversible
V_{max}	none	decrease	decrease
K_m	increase	none	increase

Table 2. Inhibitor susceptibility of proteases with different structure of catalytic site. x - L-3-carboxy-2,3-trans-epoxy-propionyl-leucylamido(guanidino)butane; xx - 3,4-dichloroisocumarine.

Proteases	Inhibitor
Aspartyl	pepstatin
Cysteinyl	E-64[x]
Seryl	3,4-DCI[xx]
Metaloproteases	1,10-phenantroline

and partly noncompetitive, binds to free enzyme and to the enzyme-substrate complex. In the Lineweaver-Burk plot, both the Michaelis constant (1/Km) and the maximum velocity (1/Vmax) increase (Fig. 1C). Mixed inhibitors hinder the formation of the enzyme-substrate complex and decrease the velocity of formation and release of the reaction product. Mixed type inhibition can be also observed when cathepsin D exerts a simultaneous effect on different substrates, including a mixture of protein partial degradation products having varied affinity for this protease [67]. The inhibitor acts competitively toward some substrates and noncompetitively toward others. Characteristic features of various types of inhibitors have been presented in Table 1.

Pepstatin acts as an inhibitor of cathepsin D and other aspartyl proteases (Table 2). It does not inhibit the activities of seryl cathepsins, cysteinyl cathepsins or metalloproteases, and their inhibitors do not block the activity of cathepsin D. A properly chosen set of inhibitors allows differentiation of cathepsin D from cathepsin A and B (Table 3), and from cathepsin E, pepsin and rennin, which are also aspartyl proteases (Table 4). Dithiotreitol, at a concentration of 1 mmol/l, pH=8.0, inactivates this cathepsin [69]. However, endogenous thiols, at physiological concentrations, do not affect cathepsin D activity.

The propeptide split off of procathepsin D during activation [70,71], α2-macroglobulin [72] and DNA fragments [73] are endogenous cathepsin D inhibitors. Their inhibitory effects are slight and observed only in special conditions. Most cathepsin D inhibitors are synthetic, peptide and polypeptide produced by microorganisms, plants and animals [67,74-77]. Specific anti-cathepsin D antibodies also have an inhibitory effect.

Table 3. The inhibitory effect of pepstatin A, antipain, leupeptine and chymostatin on cathepsins A, B and D [68].

Cathepsin	Substrate	IC_{50} (μg/ml)			
		Pepstatin A	Antipain	Leupaptine	Chymostatin
A	Cbz-L-Glu-L-Tyr	125.00	1.20	1680.00	62.5
B	Bz-l-Arg-NH_2	125.00	0.60	0.40	2.60
D	Hemoglobina	0.01	125.00	109.00	0.011

Table 4. Vulnerability of human aspartyl proteases to inhibitors. * + inhibitory effect, - lack of inhibition.

Inhibitor*	Cathepsin D	Cathepsin E	Pepsin	Renin
Pepstatin A	+	+	+	+
Ascaris sp. derived iuhibitor	-	+	+	-
Antibodies against cathepsin D	+	-	-	-

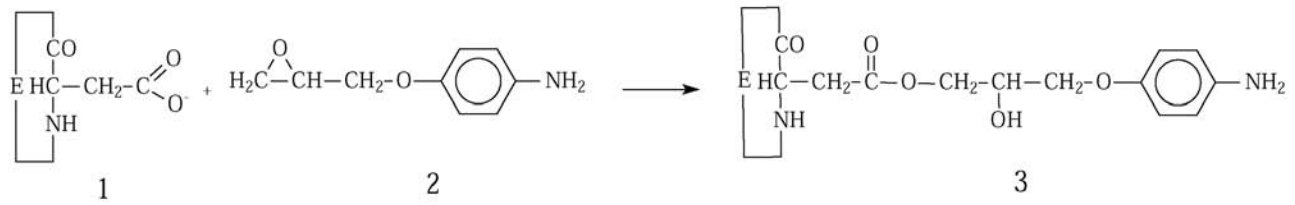


Fig. 2. Reaction of cathepsin D Asp33 ionized residue (1) with 1,2-epoxy-3-(p-nitrophenoxy)-propane (2); (3) 2-hydroksy-3-(p-nitrophenoxy)propyl ester [63].

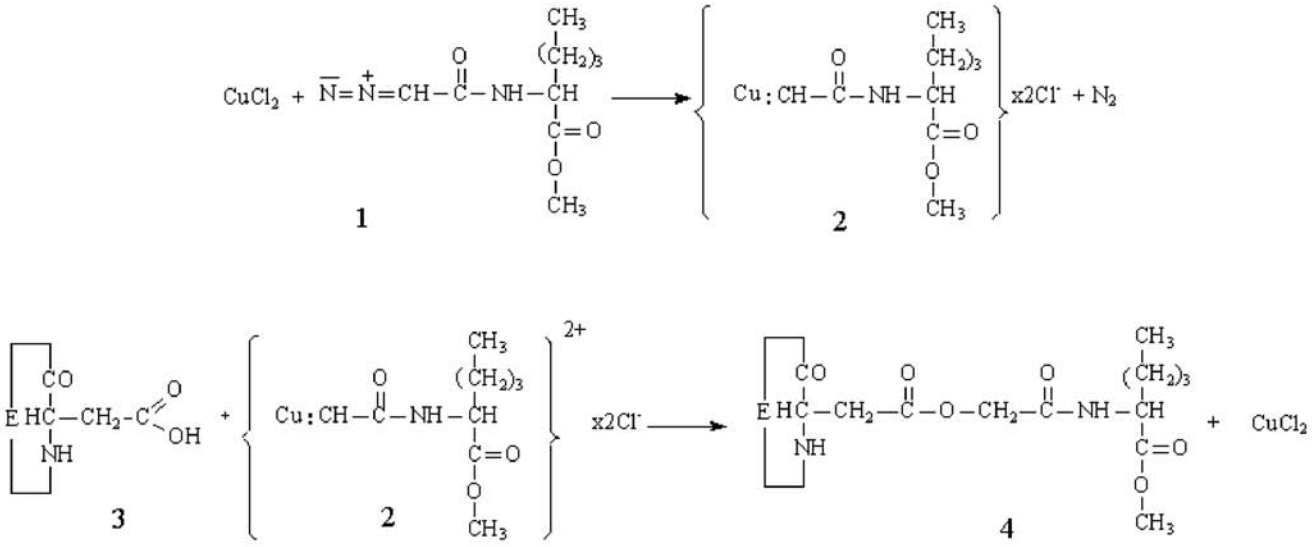


Fig. 3. Reaction of cupric chloride with diazoacetyl norleucine methyl ester (1) with creation of carbene with cupric ions complex chloride (2); nonionised Asp231 residue of cathepsin D (3) with carbene with cupric ions complex chloride (2) with creation of 2-oxo-3-ase-4-metoxycarbonyloxyl ester (4) [63].

Synthetic inhibitors

Synthetic inhibitors of cathepsin D are micromolecular organic compounds estrifying the carboxyl group of the Asp33 or Asp231 residue in the catalytic site. The Asp33 residue reacts with 1,2-epoxy-3-(p-nitrophenyloxy)-propan (Fig. 2). The compounds reacting with the Asp231 residue include diazacetyl derivatives of amino acid methyl esters. The carboxyl groups of cathepsin D are estrified by these compounds in the presence of copper ions having a catalytic function [78]. The reaction of cathepsin D with diazoacetyl norleucine methyl ester has been presented in Fig. 3. Other cathepsin D inhibitors are: diazoacetyl-glycine ethyl ester [79], diazoacetyl-phenyloalanin methyl ester [80], diazoacetyl-2,4-dinitrophenyl-ethylenediamine [81] and other diazol compounds [81-85]. The reaction of cathepsin D with diazol compounds occurs most rapidly at pH=4.5. Other cathepsin D inhibitors also include many derivatives of 4-(morpholinyl-sulphonyl)-L-Phe-P_2-(cyclohexyl)Ala[isostere]-P_1'-P_2', listed in Table V. Also ditiophosgen and 2,2-dichloro-1,3-ditiocyclobutanone [87], and methyl blue and tetranitrometane [88] have been found to inhibit cathepsin D activity. These compounds inactivate pepsin as well. However, 2,4'-dibromoacetophenone and 2-bromo-2-phenylacetophenone inhibit pepsin but not cathepsin D activity [78,89,90].

Synthetic substrate analogues

Cathepsin D activity is inhibited by structural analogues of synthetic substrates. These are oligopeptides containing at least five amino acid residues in the molecule and having L-amino acid replaced by D-amino acid. In the pentapeptide Pro1-Phe2-Phe3-Val4-Leu5, cathepsin D causes the cleavage of the Phe2-Phe3 bond [91]. Replacement of L-Leu5 residue by D-Leu5 makes this pentapeptide resistant to the action of cathepsin D and able to inhibit hydrolysis of matrix pentapeptide. In the Gly1-Phe2-Leu3-Gly4-Phe5-Leu6 hexapeptide, cathepsin D causes the cleavage of the Phe2-Leu3 bond. The hydrolysis of peptides of analogous structure but containing D-amino acids in positions P1, P3 or P4 is considerably hindered; moreover they inhibit decomposition of a hexapeptide built

Table 5. Inhibition constant (Ki) of 4-(morpholinylsulphonyl)-L-Phe-P2-(cyclohexyl)Ala(isostere)-P1-P2 derivatives, in relation to human aspartyl proteases [86]. Abbreviations: ACFHP - 4-amino-5-cyclohexyl-2,2-difluoro-3-hydroxypentanamides; AEM - 2-morpholinylethylamide; MBA - 2-methylbutylamide; ACFOP - 4-amino-5-cyclohexyl-2,2-difluoro-3-oxopentanamides; 3.4-ACDMH - 2-amino-1-cyclohexyl-3,4-dihydroxy-6-methylheptane; ACDPD - 2-(2-amino-1-cyclohexyl-3,5-dihydroxypent-6-yl)-1,3-dithiane; 3.5-ACDMH - 2-amino-1-cyclohexyl-3.5-dihydroxy-6-methylheptane; R-ACDH - 5(R)-2-amino-1-cyclohexyl-3,5-dihydroxyheptane; S-ACDH - 5(S)-2-amino-1-cyclohexyl-3,5-dihydroxyheptane.

P_2	P1-P1'	K_i nmol/l			
		pepsin	gastricsin	cathepsin D	cathepsin E
allyl	ACFHP-AEM	1240.0	>4000.0	0.8	21.0
allyl	ACFHP-MBA	27.0	13.0	3.2	0.1
allyl	ACFOP-AEM	11.0	0.9	18.0	0.5
allyl	ACFOP-MBA	24.0	9.0	0.4	0.5
allyl	3,4-ACDMH	13.0	8.0	2.5	17.0
allyl	ACDPD	16.0	>4000.0	0.6	2.2
allyl	3,5-ACDMH	57.0	19.0	0.1	2.0
allyl	R-ACDH	77.0	>4000.0	5.4	11.0
allyl	S-ACDH	69.0	>4000.0	6.1	3.3
allylthio (R/S)	S-ACDH	516.0	>4000.0	15.0	2.9
CH_2CO_2Me	R-ACDH	185.0	>4000.0	13.0	44.0
$(CH_2)_4NHCSNHMe$	S-ACDH	>10000.0	110.0	45.0	15.0
(S)–CH_2– (4–imidazole)	R-ACDH	>10000.0	>4000.0	>10000.0	850.0
(S)–CH_2–[4–(2–aminothiazole)]	R-ACDH	>10000.0	>4000.0	208.0	300.0
H	R-ACDH	>10000.0	>4000.0	>10000.0	>10000.0

Table 6. Inhibition of release of the dipeptide Gly-Phe from the substrate Gly-Phe-Leu-Gly-Phe-Leu by cathepsin D in the presence of its D-isomers [91].

D-isomer	Hydrolysis, %	Inhibition, %
Gly-Phe-Leu-Gly-Phe-Leu	100.0	0.0
Gly-Phe-Leu-Gly-Phe-D-Leu	6.0	89.2
Gly-Phe-Leu-Gly-D-Phe-Leu	3.0	73.4
Gly-D-Phe-Leu-Gly-Phe-Leu	0.0	63.1
Gly-D-Phe-Leu-Gly-D-Phe-Leu	0.0	0.0
Gly-Phe-Leu-Gly-Phe-Leu └──────────┘	0.0	0.0

up of L-amino acids. Cathepsin D vulnerability to hydrolysis and degree of inhibition by these analogues depend on the number and location of D-amino acids in the molecule (Table 6). The analogues with one D-amino acid at a maximum distance from the site sensitive to cathepsin D exert the strongest inhibitory effect. However, the analogues with two D-amino acids at the site of a cleaved Phe3-Leu3 bond and constituting a cyclic hexapeptide are not hydrolyzed and do not exhibit an inhibitory effect. This has been also confirmed by data shown in Table 7. Cathepsin D activity is also inhibited by Gly-Glu-Gly-Phe-Leu-Gly-D-Phe-Leu and aldehydes of such peptides as Ac-Leu-Leu-Nle-H, Ac-Leu-Val-Phe-H and [(s)-1-carboxy-2-phenyl-ethyl]-carbamoil-Arg-Val-Arg-H [93].

Table 7. Inhibition of cathepsin D and pepsin action by synthetic peptides [92].

Inhibitor	K_i μmol/l	
	Cathepsin D	Pepsin
pGlu-D-Phe-Pro-Phe-Phe-Val-D-Leu	0.031	0.470
D-Phe-Pro-Phe-Phe-Val-D-Leu	0.520	5.200
D-Phe-Pro-D-Phe-D-Phe-Val-D-Leu	650.000	no data
D-Phe-Pro-Phe-Phe-Val	no inhibition	no inhibition

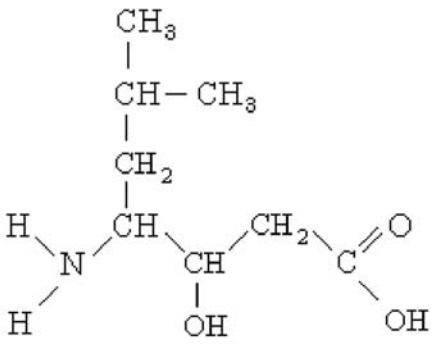


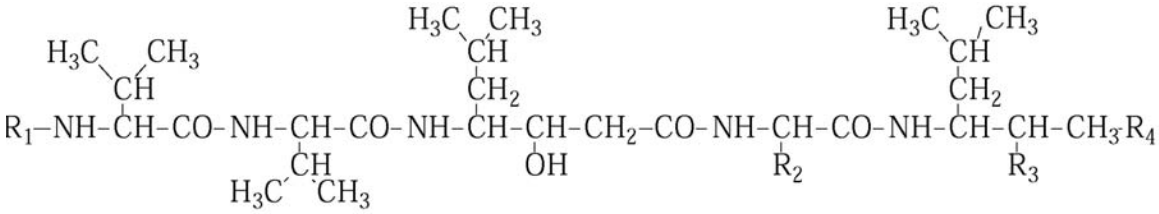
Fig. 4. Statin.

Inhibitors produced by microorganisms

Inhibitors of aspartyl proteases are synthesized by *Streptomyces testaceus*, *Streptomyces argenteolus* and other species of the genus *Streptomyces*. These inhibitors are called pepstatins as they inhibit the activity of pepsin. They also inhibit the activities of cathepsin D, cathepsin E, renin, pseudorenin, aspartyl proteases produced by microorganisms and plants [94-98].

Pepstatins are pentapeptides containing non-amino acid constituents, typical amino acids and atypical amino acid. They are composed of a sequence of 1 acyl radical, 2 valin residues, 1 statin residue, 1 alanine residue and 1 statin residue, and are shortly called Ac-L-Val-L-Val-L-Sta-L-Ala-L-Sta. Statin (Sta) is a rare atypical amino acid referred to as [(4S,3S)-4-amino-3-hydroxy-6-methylheptanoic acid] (AHMHA) (Fig. 4). Pepstatins differ from each other in the structure of the acyl radical (Fig. 5). Hydroxypepstatin and pepstanon are pepstatin derivatives with preserved inhibitory potential. Hydroxypepstatin contains serine instead of alanine. In pepstanon, at the site of C-terminal Sta, its ketone 3S-3-amino-5-methylhexanone-2 (AMHN) is located. The isovalerian acid residue is the most common acyl, hydroxypepstatin and pepstanon residue (Fig. 6). The structure of acyl residue of pepstatins depends upon culture conditions. *Streptomyces testaceus* grown in a medium containing meat extract generates isovaleryl pepstatin, whereas that cultured in a medium with casein produces hexanol-pepstatin.

The hydroxyl group Sta4 is directly involved in the inhibitory action of pepstatins, reacting with the catalytic-site residues of an aspartyl protease [102]. The Sta4 residue is a structural analogue of substrate, analogue of its transitory state and shows an inhibitory action due to structural similarity to the tetraedric immediate product



Pepstatins	R_1	R_2	R_3	R_4
A	iso-valeryl	$-CH_3$	-OH	-COOH
B	n-caproyl	$-CH_3$	-OH	-COOH
C	iso-caproyl	$-CH_3$	-OH	-COOH
D	n-heptanoyl	$-CH_3$	-OH	-COOH
E	iso-heptanoyl	$-CH_3$	-OH	-COOH
F	anteiso-heptanoyl	$-CH_3$	-OH	-COOH
G	n-capryl	$-CH_3$	-OH	-COOH
H	iso-capryl	$-CH_3$	-OH	-COOH
Hydroxypepstatine A*	iso-valeryl	$-CH_2$-OH	-OH	-COOH
Pepstanone A*	iso-valeryl	$-CH_3$	=O	-H

Fig. 5. Structure of pepstatins, hydroxypepstatin and pepstanones. *- acyl radical R1- the same as pepstatins A-H [100].

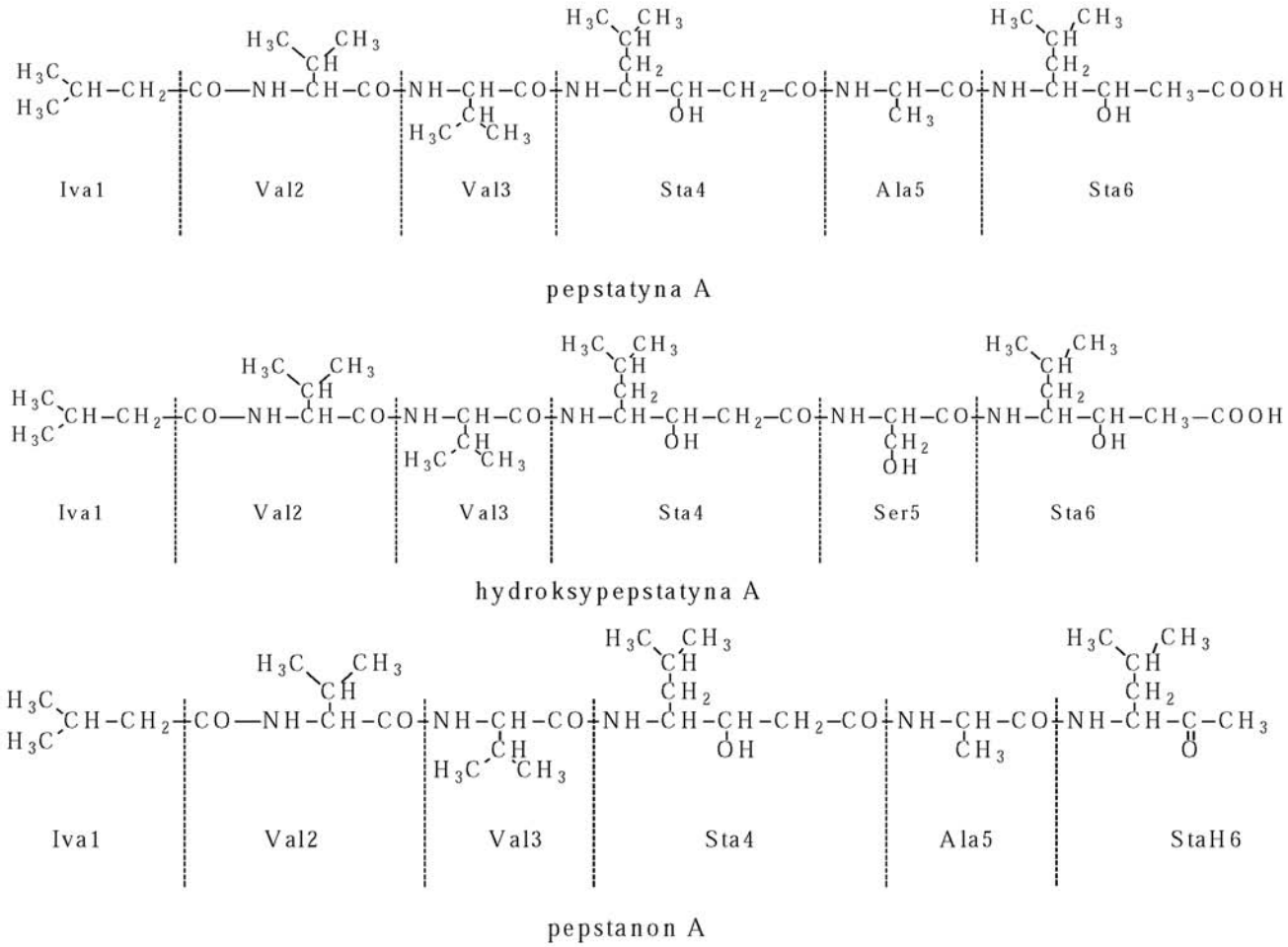


Fig. 6. Pepstatin A, hydroxypepstatin A and pepstanone A [101].

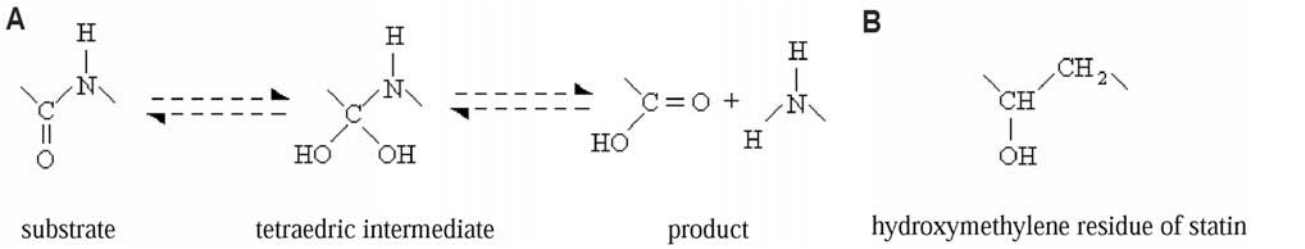


Fig. 7. Tetraedric intermediate state of the substrate (A) and stable analogue of intermediate tetraedric state which is hydroxymethylene residue (B) [103].

formed during cleavage of peptide bonds by proteases possessing carboxyl catalytic sites (Fig. 7). The mechanism of cathepsin D activity inhibition by pepstatin can be illustrated by a simplified model (Fig. 8) and a spatial model of these substances (Fig. 9). Hydrogen bonds connecting pepstatin to cathepsin D have been listed in Table 8. Pepstatin also binds to procathepsin D and inhibits its autoactivation [106].

The inhibition of aspartyl protease action by pepstatins depends to a large extent on the presence of acid residue in their structure [107]. A comparison between the inhibitory effect of pepstatins with variously structured acid residue on the activities of pepsin, cathepsin D and rennin shows that the inhibitory effects are similar in the case of the first two proteases, whereas that of rennin increases with elongation of carbon chain of the residue (Table 9). This is due to the structural similarity of the catalytic site of pepsin and cathepsin D and its different structure in the rennin molecule. Differences in the inhibitory action are also observed according to the type of the N- and C-terminal group (Table 10). The inhibitory effect of pepstatins on cathepsin D does not depend on the carbon chain length of the acid radical. Hydroxypepstatin

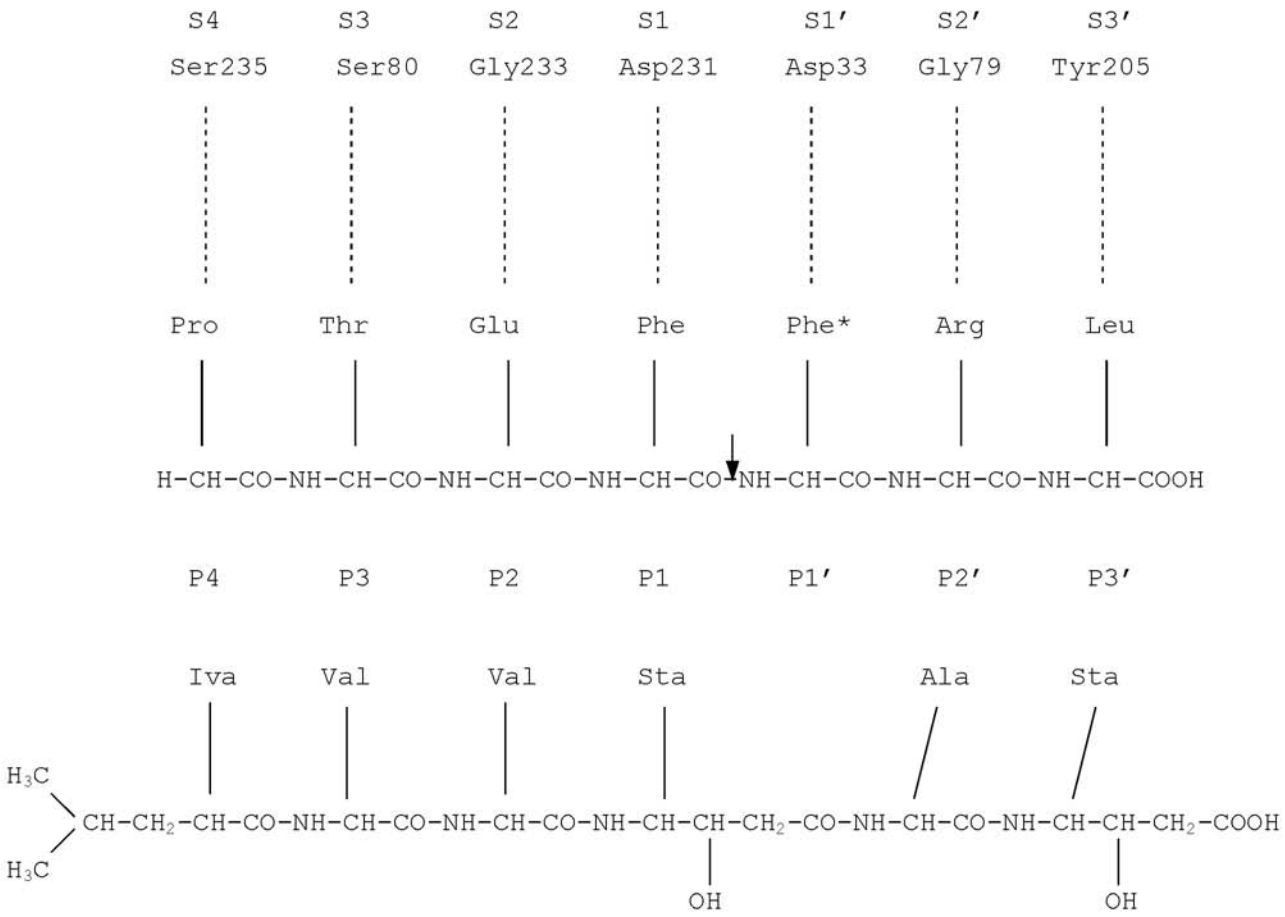


Fig. 8. Cleavage of a peptide bond -Phe4-Phe(NO2)5 by cathepsin D in a substrate and pepstatin inhibition of this process, according to Schecter and Berger [104]. S4-S3' - aminoacidic residues of catalytic site, P4-P3' - substrate or pepstatin aminoacidic residues, arrow - cleaved peptide bond, * - NO_2.

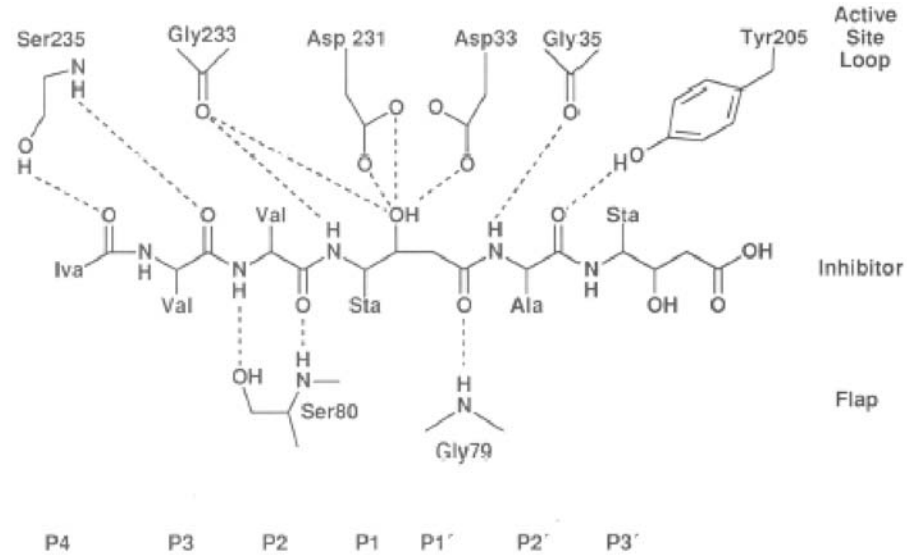


Fig. 9. Interactions between pepstatin and cathepsin D. Schematic hydrogen-bonding diagram for pepstatin bound to cathepsin D. Inhibitor side chain numbering as according to the nomenclature of Schechter and Berger, where Pn designates inhibitor side chain residues that interact with the corresponding Sn subsites within the enzyme active site. Iva and Sta indicate isovaline and statin, respectively [102,105].

exerts a similar inhibitory effect to that of pepstatins. However, the inhibitory effect of pepstanon is less pronounced. This indicates the significance of the presence of hydroxyl groups in the C-terminal fragment for the action of these inhibitors (Table 11).

The activity of cathepsin D is also inhibited by methyl ester of carbobenzoxy Z-Arg-Arg-Pro-Phe-His-Sta-Ile-His-Lys [110].

The splitting off of the Ac1 and Val2 residues by pepstatin peptidase synthesized by Bacillus Sphericus

Table 8. Hydrogen bonds between cathepsin D and pepstatin; * aminoacid residues of the active site [102].

Cathepsin D		Pepstatin		Hydrogen bond [Å]
Residue	Atom	Residue	Atom	
Ser235	Oγ	Iva1	O	2.6
Ser235	N	Val2	O	3.1
Ser80	Oγ	Val2	N	3.5
Ser80	N	Val3	O	2.9
Gly233	O	Sta4	N	2.9
Gly233	O	Sta4	OH	3.4
Asp231*	Oδ1	Sta4	OH	2.8
Asp231*	Oδ2	Sta4	OH	2.6
Asp33*	Oδ1	Sta4	OH	3.5
Asp33*	Oδ2	Sta4	OH	2.8
Gly79	N	Sta4	O	2.9
Gly35	O	Ala5	N	2.9
Tyr205	OH	Ala5	O	2.9

inactivates pepstatins (Table 12). The action of this enzyme is conditioned by the N-terminal sequence Ac1-Val2-Val3-, from which acyl radical and N-terminal valin are released. The splitting of the acyl and valin residues off pepstatin A causes a considerable reduction in the inhibitory activity towards cathepsin D, pepsin and rennin (Table 13). Addition of the acyl residue, varying according to its type, increases the inhibitory activity.

Methods in combinatorial chemistry are used in studies on modification and synthesis of new pepstatin derivatives [113-116].

Table 9. Inhibition of cathepsin D, pepsin and renin by pepstatins [101].

Pepstatin	IC_{50} µmol/l		
	Cathepsin D	Pepsin	Renin
Acetyl	9.3	1.5	24.9
Propionyl	9.1	1.5	15.2
Butyryl	8.9	1.4	9.7
iso-Valeryl	8.8	1.4	6.6
n-Caproyl	9.3	1.4	4.3
iso-Heptanoyl	9.1	1.4	2.5
n-Capryl	8.9	1.4	1.7
iso-Valeryl hydroxypepstatin	11.4	1.7	20.0
iso-Valeryl pepstanone	17.0	2.0	39.0

Table 10. Inhibition of cathepsin D, pepsin and renin activity by pepstatin and pepstatin derivatives [108].

Inhibitor	K_i µmol/l		
	Cathepsin D	Pepsin	Renin
Iva-Val-Val-Sta-Ala-Sta	0.5	0.05	13000.0
Iva-His-Pro-Phe-His-Sta-Leu-Phe-NH_2	210.0	27.0	19.0
His-Pro-Phe-His-Sta-Leu-Phe-NH_2	900.0	40.0	50.0
POA-His-Sta-Leu-Phe-OCH_3	3300.0	67.0	6300.0
POA-Leu-Sta-Leu-Phe-OCH_3	35.0	12.0	27000.0
Iva-His-Pro-Phe-His-Sta-Ile-Phe-NH_2	134.0	43.0	1.9

Table 11. Peptide aldehydes with inhibitory activity against cathepsin D [109]. Comparison with pepstatin.

Peptide aldehyde	IC_{50}. µmol/l		
	Cathepsin D	Pepsin	HIV protease
Ac-Leu-Leu-Nle-H	35.0	24.0	5.2
Cbz-Ile-Phe-H	20.0	>200.0	4.8
Me-CO-Val-Phe-H	40.0	>200.0	5.4
N-Ac-Leu-Val-Phe-H	37.0	100.0	0.9
Iva-Val-Val-Sta-Ala-Sta-OH	<0.04	<0.005	2.0
Iva-Val-Val-Sta-Ala-Sta-H	17.0	2.0	3.0

Table 12. Substrate specificity of pepstatin hydrolase [111].

Substrates	Relative activity [%]	Ninhydrin-positive products
iso-Valeryl-Val-Val-AHMHA-Ala-AHMHA (pepstatin A)	100	Val, Val-AHMHA-Ala-AHMHA
n-Caproyl-Val-Val-AHMHA-Ala-AHMHA (pepstatin B)	723	Val, Val-AHMHA-Ala-AHMHA
Acetyl-Val-Val-AHMHA-Ala-AHMHA (pepstatin Ac)	250	Val, Val-AHMHA-Ala-AHMHA
iso-Valeryl-Val-Val-AHMHA-Ser-AHMHA (hydroxypepstatin A)	55	Val, Val-AHMHA-Ser-AHMHA
iso-Valeryl-Val-Val-AHMHA-Ala-3-amino-5 methylhexanone-2	2	Val, Val-AHMHA-Ala-3-amino-5-methyl-hexanone-2
Acetyl-Val-AHMHA-Ala-AHMHA	0	None
iso-Valeryl-Val-AHMHA-Ala-AHMHA	0	None
Benzoyl-Val-AHMHA-Ala-AHMHA	0	None
Phenoxyacetyl-Val-AHMHA-Ala-AHMHA	0	None
2-Phenoxypropionyl-Val-AHMHA-Ala-AHMHA	0	None
iso-Valeryl-Val	0	None
iso-Valeryl-Val-Val	0	None
iso-Valeryl-Val-Val-AHMHA	55	Val, Val-AHMHA
iso-Valeryl-Val-Val-AHMHA-Ala-AHMHA	100	Val, Val-AHMHA-Ala-AHMHA
Val-Val-AHMHA		Val, Val-AHMHA

Table 13. Inhibitory activities of Val-AHMHA-Ala-AHMHA, its N-acylated derivatives and pepstatin A against cathepsin D, pepsin and renin [112].

Compounds	ID_{50} μg/ml		
	Cathepsin D	Pepsin	Renin
Val-AHMHA-Ala-AHMHA	6.5	10.0	>250
Acetyl-Val-AHMHA-Ala-AHMHA	0.42	0.031	>250
iso-Butyryl-Val-AHMHA-Ala-AHMHA	0.28	0.021	>250
iso-Valeryl-Val-AHMHA-Ala-AHMHA	0.05	0.01	>250
Palmitoyl-Val-AHMHA-Ala-AHMHA	1.1	0.45	>250
Benzoyl-Val-AHMHA-Ala-AHMHA	0.05	0.031	>250
Phenoxyacetyl-Val-AHMHA-Ala-AHMHA	0.008	0.02	31
2-Phenoxypropionyl-Val-AHMHA-Ala-AHMHA	0.01	0.02	-
iso-Valeryl-Val-Val-AHMHA-Ala-AHMHA (pepstatin A)	0.011	0.01	4.5

Polypeptide plant inhibitors

Polipeptide inhibitors of aspartyl proteases can be found in spare plant organs such as seeds, bulbs and fruits, and more seldom in vegetative organs like leaves, roots and flowers. They protect plant proteins against uncontrolled actions of endogenous and exogenous proteases synthesized by parasitic viruses, bacteria, moulds and insects [117-121].

Well defined amino acid residues constituting the inhibitory reactive site, complementary to the amino acid residues of the protease catalytic site, are found on the surface of the polypeptide inhibitor molecule. The type of amino acid residues of this site determines the group and individual specificity of the inhibitor. This can be a sequence specific to the catalytic sites of aspartyl proteases or to the catalytic site of a particular protease, also cathepsin D. The inhibitor forms a permanent complex with an in the 1:1 ratio and blocks the action of cathepsin due to hydrophobic and ionic hydrogen bonds of the reactive site of the inhibitor, the catalytic site of cathepsin and their closest environment.

Potato inhibitors of cathepsin D

Potato bulbs contain six or eight polypeptide inhibitors of cathepsin D, depending on the species [105,122-

```
Potato1 MMKCLFLLCLCLLPIVVFSSTFTSQNLIDLPSESPVPKPVLDTNGKELNPNSSYRIISIG   60
Potato2 MMKCLFFLCLCLFPILVFSSTFTSQNPINLPSESPVPKPVLDTNGKKLNPNSSYRIISTF   60
Potato3 MMKCLFLLCLCLVPIVVFSSTFTSQNPIDLPSESPLPKPVLDTNGKELNPNSSYRIISIG   60
Potato4 ------------------------------ESPLPKPVLDTNGKELNPNSSYRIISIG   28
Potato5 MMKCLFLLCLCLlPIVVFSSTFTSQNPIDLPSESPLPKPVLDTNGKELNPNSSYRIISIG   60
Potato6 ------------------------------------------------NSSYRIISIG   10
Potato7 ------------------------------------------------NSSYRIISIG   10
Potato8 ------------------------------ESPLPKPVLDTNGKELNPNSSYRIISIG   28
                                                            ********

Potato1 RGALGGDVYLGKSPNSDAPCPDGVFRYNSDVGPSGTPVRFIPLSTNIFEDQLLNIQFNIP  120
Potato2 WGALGGDVYLGKSPNSDAPCPDGVFRYNSDVGPSGTPVRFIPLSTNIFEDQLLNIQFNIP  120
Potato3 RGALGGDVYLGKSPNSDAPCPDGVFRYNSDVGPSGTPVRFIPLSGGIFEDQLLNIQFNIP  120
Potato4 RGALGGDVYLGKSPNSDAPCPDGVFRYNSDVGPSGTPVRFIPLSGGIFEDQLLNIQFNIA   88
Potato5 RGALGGDVYLGKSPNSDGPCPDGVFRYNSDVGPSGTFVRFIPLSGGIFEDQLLNIQFNIA  120
Potato6 RGALGGDVYLGKSPNSDAPCPDGVFRYNSDVGPSGTPVRFIPLSGGIFEDQLLNIQFNIP   70
Potato7 RGALGGDVYLGKSPNSDAPCPDGVFRYNSDVGPSGTPVRFIPLSTNIFEDQLLNIQFNIP   70
Potato8 AGALGGDVYLGKSPNSDAPCPDGVFRYNSDVGPSGTPVRFIPLSGGIFEDQLLNIQFNIP   88
         ****************.****************** ******* .*************.

Potato1 TVKLCVSYTIWKVGNLNAHLRTMLLETGGTIGQADSSYFKIVKSSKFGYNLLYCPITR-H  179
Potato2 TVKLCVSYTIWKVGNLNTHLWTMLLETGGTIGKADSSYFKIVKSSKFGYNLLYCPITRPP  180
Potato3 TVRLCVSYTIWKVG-INAYLRTMLLETGGTIGQADSSYFKIVKSSILGYNLLYCPITR-P  178
Potato4 TVKLCVSYTIWKVGNLNAYFRTMLLETGGTIGQADSSYFKIVKLSNFGYNLLYCPITP-P  147
Potato5 TVKLCVSYTIWKVGNLNAYFRTMLLETGGTIGQADSSYFKIVKLSNFGYNLLYCPITP-P  179
Potato6 TVKLCVSYTIWKVGNLNAYFRTMLLETGGTIGQADNSYFKIVKSSKIGYNLLSCPFTS--  128
Potato7 TVKLCVSYTIWKVGNLNAYFRTMLLETGGTIGQADNSYFKIVKSSKIGYNLLSCPFTS--  128
Potato8 TVKLCVSYTIWKVGNLNAYFRTMLLETGGTIGQADNSYFKIVKLSNFGYNLLSCPFTS--  146
         **:*********** :*::: ***********:**.******* * :***** **:*

Potato1 FLCPFCRDDNFCAKVGVVIQNGKRRLALVNENPLDVLFQEV  220
Potato2 IVCPFCRDDDFCAKVGVVIQNGKRRLALVNENPLDVLFQEV  221
Potato3 ILCPFCRDDDFCAKVGVVIQKGKRRLALVNENPLDVNFKEV  219
Potato4 FLCPFCRDDNFCAKVGVVIQNGKRRLALVNENPLDVLFQEV  188
Potato5 FLCPFCRDDNFCAKVGVVIQNGKRRLALVNENPLDVLFQEV  220
Potato6 IICLRCPEDQFCAKVGVVIQNGKRRLALVNENPLDVLFQE-  168
Potato7 IICLRCPEDQFCAKVGVVIQNGKRRLALVNENPLDVLFQEV  169
Potato8 IICLRCPEDQFCAKVGVVIQNGKRRLALVNENPL-------  180
         ::*  * :*:**********:*************
```

Fig. 10. Aminoacid sequences of aspartic protease inhibitor from potato. * - identical amino acid residues; **:.** - conserved amino acid residues [132].

131]. They show a considerable similarity in the amino acid composition, sequence (Fig. 10) and in the number and location of disulphide bridges. Potato inhibitors possess two inhibitory sites: one binding cathepsin D and the other binding trypsin [133], and they do not inhibit the activity of pepsin. Inhibitors of aspartyl proteases also occur in potato leaves [134].

Inhibitors from pumpkin fruit

Cathepsin D inhibitor, which also inhibits the activity of pepsin and acid protease from fungus *Glomerella cingulata* has been isolated from pumpkin fruit [135]. It is composed of 96 amino acid residues and occurs in three molecular forms differing slightly in the amino acid sequence. Two molecular isoforms are found to predominate (Table 14) and are called DNIS and HDVA due to differences in the amino acid sequence (Fig. 11). The sequence, however, is very similar, especially in the N-terminal sequence (MGPGPAIGEVIG) and in the internal sequence (FYNVVVLEK). These inhibitors do not contain cysteinyl residues or sugar constituent. The molecular mass of DNIS isoform is 10551 Da, and of HDVA isoform - 10527 Da. These isoforms constitute a dimeric form with a molecular mass of 21000 (10551 + 10527). During purification, the dimeric form breaks down and monomers preserve their activity. The inhibitor is permanent in an acid and neutral environment, but sensitive to alkalization and elevated temperature.

Table 14. Aminoacidic composition of DNIS and HDVA inhibitors isolated from pumpkin fruit [135].

Aminoacid	DNIS	HDVA
S	7	6
G	7	7
H	3	4
R	1	1
T	0	0
A	8	9
P	6	6
Y	3	3
V	8	9
M	1	1
I	10	9
L	9	9
F	5	5
K	7	7
W	1	1

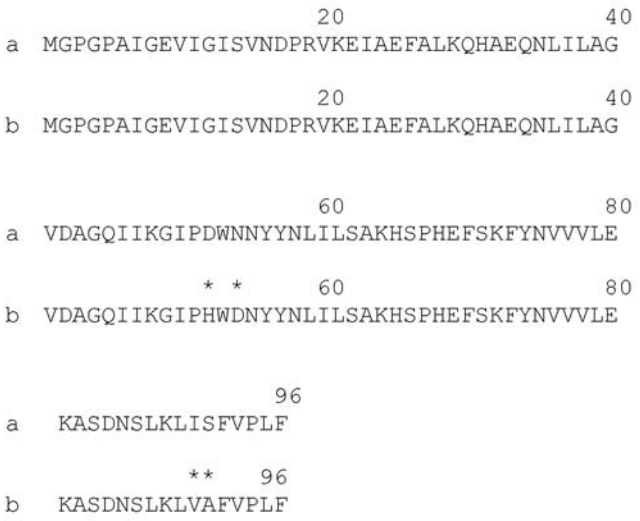


Fig. 11. Amino acid sequences of DNIS (a) and HDVA (b) isoforms of squash aspartic proteinase inhibitor [135].

Inhibitors synthesized by other plants

Cathepsin D inhibitors can be found in the seeds of lentil and vicia [136], but are not found in the seeds of 25 cereal species and pulses consumed by man. Cathepsin D inhibitors have been isolated from the seeds of wheat [137], barley [138], tomato leaves [139-141], tobacco leaves [142], aubergine fruit (*Solanum melongea*) and from trailing nightshade leaves (*Solanum dulcamara*) and black nightshade (*Solanum nigrum*). The inhibitor obtained from tomato leaves inhibits the activity of the proteases listed in Table 15. The effects of cathepsin D inhibitors isolated from tomato leaves and potato bulbs have been compared in Table 16.

The synthesis of plant cathepsin D inhibitors is stimulated by mechanical tissue injury [144], as well as jasmonic acid, methyl ester of jasmonic acid and abscisic acid [145-149]. Salicylic acid inhibits synthesis of these inhibitors [150].

Techniques of molecular biology have been used to obtain plant cathepsin D inhibitors [146,151,152].

Table 15. Inhibitor isolated from tomato leaves and inhibitory effect on different aspartyl proteases [143]. All measurements were performed in pH=4.7.

Aspartyl proteinase	Ki, nmol/l*
Bovine chymosine	>1200
Cyprosine	>1200
Endotiapepsin	>2400
Human gastricsin	>1200
Cardosin B	>2400
Human cathepsin D	260
Human cathepsin E	>1200
Penicyllopepsin	>1200
Human pepsin	>2400
Yeast proteinase A	30
Proteinase HIV-1	>1000

Inhibitors synthesized by lower animals

Cathepsin D activity is inhibited by equistatin, a protein inhibitor isolated from *Actinia equina* [153,154]. Equistatin inhibits the activity of cystein proteases: papain, cathepsin B, cathepsin L and cathepsin D, but not the activity of pepsin, chymotrypsin or HIV protease. Equistatin has a domain structure (Fig. 12).

Table 16. Inhibition of cathepsin D activity indifferent pH by inhibitor isolated from tomato leaves and potato bulbs [143].

Inhibitor	pH	Cathepsin D Ki, nmol/l
Isolated from potato	3.1	30.0 ± 5.0
	4.7	5.0 ± 0.5
Isolated from tomato	3.1	580.0 ± 100.0
	4.7	260.0 ± 30.0

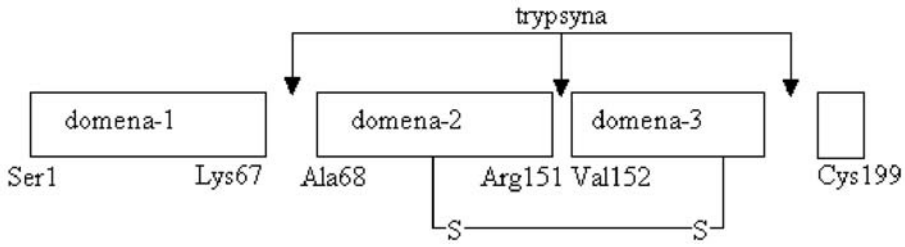


Fig. 12. Schematic diagram of equistatin [154].

Table 17. Inhibition constants (Ki) of aspartyl proteases and papain with domain-1, domain-2, 3 and equistatin [154].

Protease	Inhibitor	Ki, nmol/l
Cathepsin D	equistatin	0.30
	domain-1	>1000.00
	domain-2, 3	0,40
Pepsin	equistatin	>1000.00
Chymosine	equistatin	>1000.00
HIV protease	equistatin	>1000.00
Papain	equistatin	0.57
	domain-1	0.61
	domain-2, 3	>1000.00

Table 18. Kinetic constants (Ki) for the inhibition of aspartic proteases by the Ascaris suum inhibitor [207].

Protease	pH	Ki, nmol/l
Pepsin	4.0	2.6
Cathepsin E	3.0	3.4
Cathepsin D	4.0	$>10^4$
Renina	6.0	$>10^4$

Trypsin digestion of equistatin leads to the formation of domain-1 and domain-2 and 3, which are connected by the disulphate bridge. These domains originate from cleavage of the peptide bonds Lys67-Ala68 and Arg151-Val152. Trypsin also splits the inactive C-terminal fragment off domain-3. Domain-1 inhibits the activity of cystein proteases (papains), whereas domain-2 and domain-3 inhibit the activity of cathepsin D (Table 17).

Ascaris lumbricoides and *Ascaris suum* synthesize the inhibitors which block the activity of cathepsin E, pepsin and rennin but not cathepsin D [155-158]. They are used to differentiate the activities of these proteases in the nonfractionated material. The activity of the inhibitor from Ascaris suum towards aspartyl proteases has been presented in Table 18.

Endogenous cathepsin D inhibitors

Endogenous cathepsin D inhibitors include α2-macroglobulin and polyanion compounds - glycozaminoglycans and deoxyribonucleic acids.

The α2-macroglobulin (α2-M), a 725,000-Da glycoprotein, is built up of four structurally, electrophoretically and immunologically identical 185,000-Da subunits. Each subunit is composed of 1,451 amino acid residues and contains 8 oligosaccharide chains. The subunits combine to form dimers by means of disulphide bonds Cys225-Cys408 and Cys-447-Cys540. The dimers attached to hydrogen- and hydrophobic-bonds form a tetramer. The α2-M differs from typical inhibitors in protease binding and low specificity, and it shows a two-stage interaction with proteases. In the first stage, protease recognizes a specific peptide bond in the central fragment of the polypeptide chain of each subunit with approximately 45 amino acid enzymes [160,161]. In this fragment, peptide bonds formed by residues of 15 amino acids meet the specificity requirements of each endopeptidase ("bait" principle) [162]. Therefore, the α2-M is a universal broad-spectrum inhibitor, reducing the activity of all four endoprotease classes [163-165]. In the case of cathepsin D, the -Phe684-Tyr685- bond undergoes cleavage. However, the α2-M exerts no effect on the activity of exopeptidases, as the peptide bond situated within the chain cannot be cleaved by aminopeptidases, carboxypeptidases, tripeptidases and dipeptidases. Specific peptide bond cleavage causes a rapid change in the conformation of all subunits and protease arrest in stage two ("trap" principle). Thus, protease becomes inaccessible to protein substrates and macromolecular inhibitors. Immediate conformatory changes in both α2-M subunit pairs block the binding of the second protease molecule. It means that only one α2-M molecule binds only one protease molecule. The other protease molecule can be bound by α2-M following cleavage of the peptide bond in the second pair of subunits, prior to conformatory changes initiated by the first molecule. Then, the two protease molecules are bound at two independent but equivalent sites of the α2-M molecule, which may occur only at a high concentration of a relatively low molecular mass protease, e.g. trypsin or chymotrypsin, but not in the case

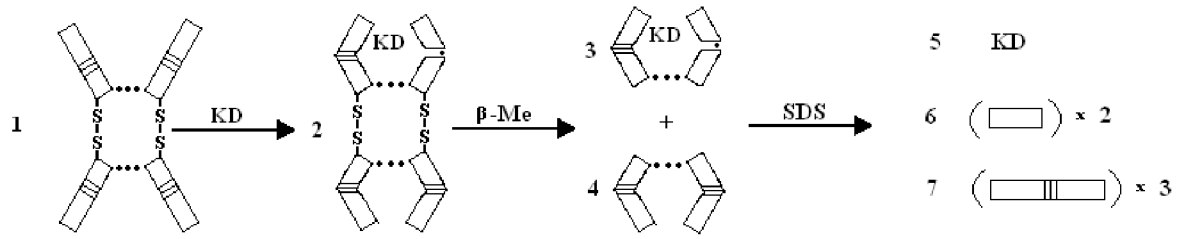


Fig. 13. Schematic structure of α2-macroglobulin binding with cathepsin D (KD) and action of β-mercaptoethanol (β-Me) and dodecyl sulphate (SDS) on inhibitor-cathepsin D complex. ≡ - "bait" region, 1 - tetramer, 2 - tetramer-cathepsin D complex, 3 - dimer-cathepsin D, 4 - dimer, 5 - cathepsin D, 6 - fragments of monomer, 7 - monomers.

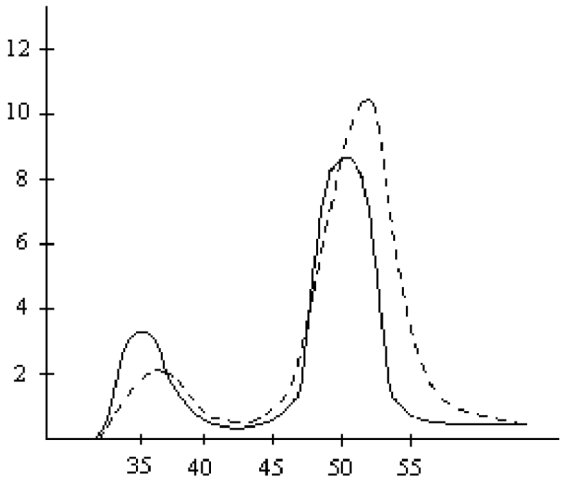


Fig. 14. Gel chromatography of ^{125}I-labelled cathepsin D [169]. Cathepsin D and α2-macroglobulin - solid line, cathepsin D with pepstatin and α2-macroglobulin - stroked line.

of cathepsin D that occurs in low concentrations in body fluids and possesses a relatively high molecular mass. The reaction of α2-M with cathepsin D takes place according to the following scheme: α2-M + KD → α2-Mx + KD → α2-Mx-KD. In physiological conditions, cathepsin D binding to α2-M is irreversible. The use of depolimerizing chemical compounds allows observation of the course of cathepsin D-induced changes in the structure of α2-M subunits (Fig. 13). β-mercaptoethanol (1%) cleaves tetramers to form a complete dimer and a dimer built up of a full dimer and one half of the monomer whose other half has been released. Dodecylosulphate (0.1%) releases nonconvalently bound complete and incomplete monomers. The catalytic site of cathepsin D, occupied by α2-M, is free. Micromolecular substrates and micromolecular inhibitors have access to this site, whereas the macromolecular ones do not [166]. The α2-M inhibits the residual activity of cathepsin D at pH=6.0-6.2, but not at a lower pH, because of structural instability of this protease [167,168]. Approximately 20% of plasma cathepsin D is blocked by α2-M [169]. The remaining 80% is the procathepsin D, the inactive precursor which does not interact with α2-M. The above data were obtained by means of Sephadex G-75 gel chromatography using ^{125}J-labeled cathepsin D (Fig. 14). Pepstatin blocks the interaction between cathepsin D and α2-macroglobulin, due to which the amount of cathepsin D bound to the inhibitor is reduced to approximately 8%. The α2-M is synthesized in fibroblasts and then passes to the intracellular fluid, lymph and blood [170]. In the plasma, α2-M occurs at a concentration of 260.0 mg/100 ml (3.3 μmol/l), constituting approximately 0.4% of plasma proteins [171]. The physiological role of α2-M is to prevent proteins against uncontrolled proteolysis, which takes place through binding, activity inhibition and removal of proteases from blood plasma and intracellular fluid. The α2-M-protease complexes are captured by membrane receptors of macrophages and hepatocytes [172], undergo endocytosis and are transported to lysosomes. Then, the complexes undergo digestion and the receptors return to the cell surface. The antiproteolytic effect of α2-M is counteracted by methylamine [173].

Cathepsin D reduces formation of ion complexes with glycozaminoglycans [174] and polysaccharides [98]. The strongest inhibitory effect is exerted by heparin (52%), weaker by chondroitin sulphate (36%) and the weakest by hialuron acid (10%). Reduction in cathepsin D activity by glycozaminoglycans is pH-dependent. The greatest decrease can be observed at pH=4.0-4.5, whereas at a lower pH, the inhibitory effect is reduced. The inhibitory effect of glycozaminoglycans is counteracted by alkaline proteins.

The activity of cathepsin D is also reduced by formation of ion complexes with 18b and 53b DNA fragments [73]. There is a correlation between the inhibitory activity of cathepsin D and the melting temperature (Tm) of a DNA molecule. These fragments also decrease the activity of cathepsin E, but not the activity of seryl and cysteine proteases.

Antibodies

Cathepsin D (antigen) introduced parenterally to another species induces production of antibodies (anti-enzymes). Antigen determinants and catalytic sites of

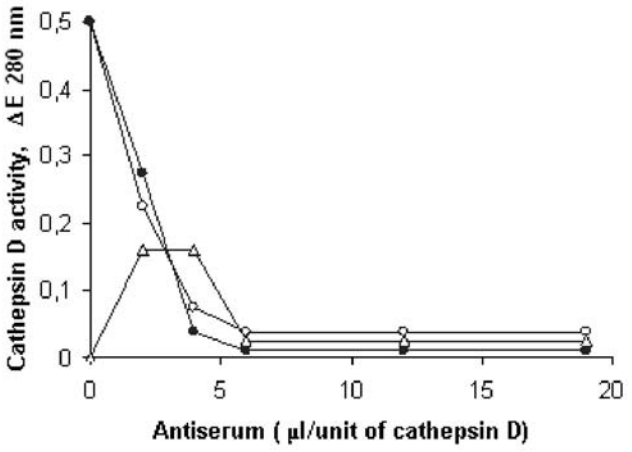


Fig. 15. Effect of antiserum ratio on cathepsin D activity in pH=5.5 [176]. o - before centrifugation, ● - in supernatant, ▲ - in precipitate.

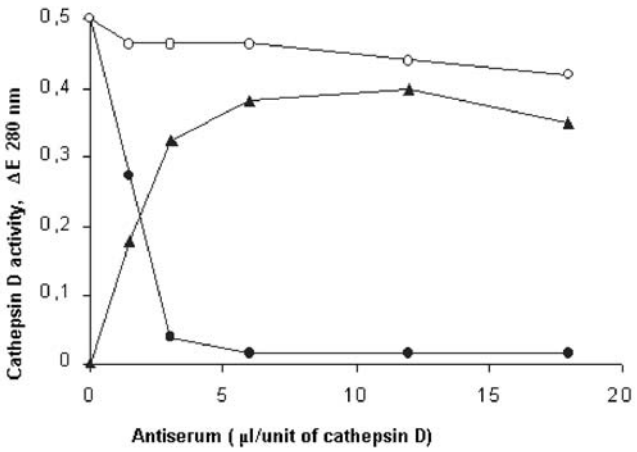


Fig. 16. Effect of antiserum ratio on cathepsin D activity in pH=3.2 [176]. o - before centrifugation, ● - in supernatant, ▲ - in precipitate.

cathepsin D are located at adjacent sites in a molecule. The cathepsin D binding to an antibody hinders access of substrate to the catalytic site [175]. The site is larger for the macromolecular than it is for the micromolecular substrate. The inhibitory effect also depends upon the chemical structure of a substrate, being more pronounced for hemoglobin than for proteoglycans [176]. The cathepsin D has a few sequential and conformatory antigenic determinants against which antibodies are produced. The inhibitory effect has been shown to be exerted by antisera and isolated antibodies. Anti-cathepsin D antibodies have been obtained from Sepharose 4B-bound cathepsin D using an immunosorption method. The inhibitory effect is also found to be exerted by monovalent fragments of antigen binding (Fab) of immunoglobulins, due to antibody fragmentation with papain or pepsin and distribution of the products obtained in Sephadex G-75 [177,178]. Antibodies are the most specific cathepsin D inhibitors. Their specificity is absolute, referring not only to cathepsin D but also to its species-related origin. Antibodies directed against cathepsin D isolated from a particular organ also inhibit the action of this cathepsin isolated from other organs of the same species, but do not react with cathepsin D from other species. Complete inhibition of cathepsin D activity occurs at an approximately six-fold molar excess of antibodies. The formation of the cathepsin D-antibody complexes is most intense at pH=5.0 [179]. It is then that cathepsin D solubility decreases and shedding from the solution takes place. The activity of cathepsin D measured at pH=5.0 with the use of hemoglobin is almost completely hampered in a reactive mixture prior to centrifugation, in supernatant and sediment (Fig. 15). However, the activity of cathepsin D is not inhibited by antiserum at pH=3.2 (Fig. 16). No activity can be found in the supernatant, whereas in the sediment the activity is nearly the same as with noncentrifuged material. Thus, due to immunoinhibition, there is a shift in the optimum pH of cathepsin D to acid direction [176]. The effect of immunocomplex formation on the action of cathepsin D on proteoglycans has been shown in Fig. 17. Conformatory changes in the cathepsin D complexed with antibody stabilize the structure and cause an increase in its thermoresistance. The antibodies against lysosomal proteases accumulate in cultured fibroblast lysosomes [180].

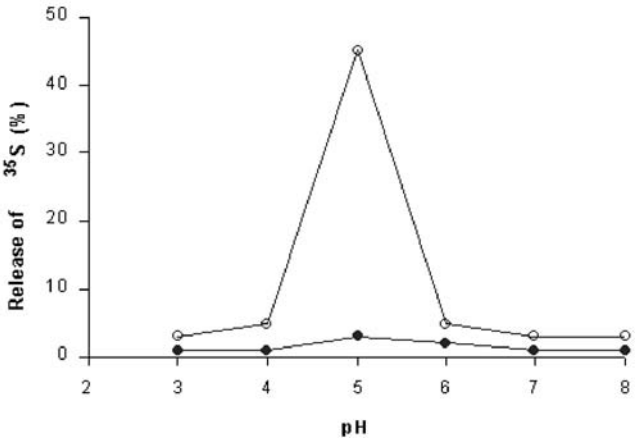


Fig. 17. Inhibitory effect of antiserum on cathepsin D activity against ^{33}S-proteoglicans. ● - cathepsin D, ▲ - cathepsin D and antibody [176].

The biological role and application of cathepsin D inhibitors

The acid pH of lysosomes has a basic role in the activation of procathepsin D and activity of cathepsin D. The propeptide released during autoactivation of procathepsin D, which at weakly acid pH binds electro-

Table 19. Distribution of ^{3}H-pepstatin after enteral introduction in mice [201].

Organ	Time/μg			
	2 h	6 h	12 h	24 h
Colon	63.732	71.338	1.479	0.037
Stomach	17.338	13.380	0.135	0.101
Jejunum	11.224	5.900	0.352	0.178
Urine	2.097	2.963	4.304	5.803
Skeleton	0.418	0.455	0.276	0.229
Skin	0.381	0.426	0.262	0.262
Liver	0.247	0.205	0.170	0.130
Muscles	0.244	0.316	0.170	0.039
Plasma	0.036	0.088	0.075	0.071
Erythrocytes	0.009	0.034	0.038	0.029
Faeces	0.0	3.754	84.879	77.178
Other organs	0.251	0.344	0.417	0.246
Summary	95.977 (96.5%)	99.203 (99.9%)	92.557 (93.0%)	84.303 (85.0%)

statically to cathepsin D and inhibits its activity, also plays a certain regulatory part [70,71]. In the case of lysosomal damage and cathepsin D translocation to the cytosol of the intercellular fluid and plasma, neutral pH and α2-macroglobulin prevent uncontrolled proteolysis [181].

Exogenous cathepsin D inhibitors play a key role in identification of the structure of the catalytic site of this protease [57]. The binding of a radioisotope-labeled inhibitor to the catalytic site of cathepsin D, limited proteolysis and isolation of a fragment containing a marker allow determination of the composition and the amino acid sequence of the site. A comparison of differences in sensitivity to inhibitors facilitates differentiation of aspartyl proteases. The activities of cathepsin E and pepsin are found to be blocked by aspartyl protease inhibitor from human ascaris, whereas cathepsin D and renin are insensitive to this inhibitor.

Cathepsin D inhibitors have analytical and preparative significance. Pepstatin has been used for quantitative determination of this protease by catalytic site titration, whereas Dansyl-pepstatin for cathepsin D catalytic site titration [182]. Lysosomal protease inhibitors are added to the solutions used to obtain subcellular fractions and fractionation of cell proteins in order to prevent proteolytic degradation. High specificity of binding to inhibitors allows isolation of cathepsin D by means of affinity chromatography, using inhibitors [20,183-186] and constant carrier-bound antibodies. Differences in the pH values between procathepsin and cathepsin D bonds with pepstatinyl-agarose help separate these two forms of the enzyme [187,188]. The constant carrier-bound cathepsin D is used to isolate its inhibitors [189]. The carrier-bound propeptide allows cathepsin isolation [190].

Pepstatin is used to determine the function of the cathepsin D found in a mixture with other cathepsins in protein degradation that occurs in the cell [191,192]. Inhibition of cell proliferation by pepstatin confirms the hypothesis that this process is stimulated by cathepsin D. The use of specific antibodies against cathepsin D has shown its significant involvement in degradation of macrophage proteins and cartilage proteoglycans [193]. Cathepsin D is localized within cells and tissues by means of specific fluorescein-labeled antibodies [20]. Methylmercury derivatives of pepstatin have been used for intracellular localization of cathepsin D [194]. The immunochemical method allows differentiation between cathepsin D and cathepsin E [195].

Attempts have been made to use cathepsin D inhibitors for therapeutic purposes. Synthetic inhibitors, due to their high toxicity, cannot be used for these purposes. Natural inhibitors can regulate proteolysis velocity in vivo. Most studies have dealt with the therapeutic application of pepstatin. Pepstatin has been used in pathological conditions occurring with increased cathepsin D activity, such as inflammatory states, burns, gastric ulcer, hypertension and Shwartzmann - Sanarelli phenomenon [99, 103, 132, 196-200]. Its advantage consists in low toxicity and low molecu-

lar weight, which permit cell and tissue penetration. Pepstatins can be administered orally or intraperitoneally. Pepstatin dose of DL_{50} is very high - in rats 2.0 g/kg of body weight for oral administration and 0.875 g/kg for intraperitoneal administration [200]. Pepstatin distribution in mouse organs has been presented in Table 19. Low toxicity of pepstatin allows it to be used to investigate processes dependent on cathepsin D effect on culture cells [202]. Anti-cathepsin D antibodies have been also used to inhibit proteolysis in vivo. Antibodies are obtained by cells via pinocytosis and accumulated in lysosomes. This causes inhibition of degradation of the hemoglobin added to a macrophage culture. Antibodies have no effect on cell viability or pinocytosis.

One inhibitor cannot inactivate all proteases of the lysosomal extract. Only a mixture of inhibitors, each inactivating protease of different catalytic site structure, may inhibit cell proteolysis [203]. A similar effect can be achieved thanks to polyvalent immune serum obtained after administration of the lysosomal extract to experimental animals. The immune serum added to a fibroblast culture was stored in lysosomes, which showed lower activity of cathepsins, especially cathepsin D and reduced exogenous protein degradation potential. Pinocytosis was impaired and lysosomes enlarged.

Determination of concentration, activity and tissue and cell location of cathepsin D inhibitors

The activity of cathepsin D is determined using a test, in which: 1 - enzyme releases the reaction product in an amount corresponding to the absorbance of 0.5; 2 - inhibitor inhibits 50% of enzyme activity. The inhibitor activity is calculated according to the formula: inhibitor, U/ml/min = (d x r) : t, in which: d means difference in the amount of the reaction products in a test without and with inhibitor, r - inhibitor dilution, t - time of incubation. Determination of the effect of the preincubation time of protease with inhibitor on the measurement outcome allows detection of its immediate or progressive action. A synthetic cathepsin D substrate, namely Pro-Thr-Glu-Phe-Phe(NO_2)-Arg-Leu should be used for determinations [104]. The use of protein substrate (hemoglobin) is not recommended due to the fact that indirect degradation products undergo further digestion and final degradation products may inhibit protease activity. Measurement of the kinetic constants in the presence of many substrates whose concentrations are varied may be biased.

The inhibitory constant dimension (Ki), *i.e.* enzyme-inhibitor dissociation constant, is the measure of inhibitor activity; the lower the Ki value, the higher the affinity of inhibitor activity for the enzyme [204].

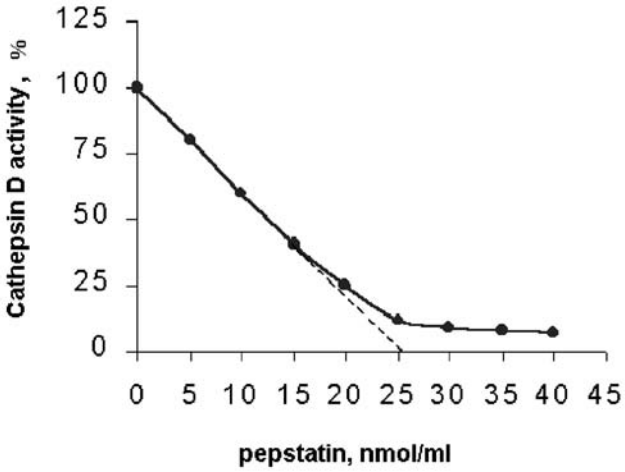


Fig. 18. Titration of the cathepsin D active site [207].

To compare the suppressive actions of various inhibitors, their concentrations that reduce cathepsin D activity by 50% (ID_{50}), expressed in μm/1 l, are determined [204]. The enzyme-inhibitor molar ratio is determined from a plot of cathepsin D activity correlation (in %) as a function of enzyme-inhibitor molar ratio [205,206] (Fig. 18). The curve bends at higher inhibitor concentrations due to partial dissociation of the complex. The inhibitor concentration that causes a 50% enzyme activity reduction is used for analysis. Prior to the therapeutic application of the inhibitor, its toxicity expressed by DL_{50} value should be determined. The activity of α2-M is measured through the assessment of proteolytic activity reduction in a standard testing system. The α2-M concentration is assayed, using antibodies, by the nephelometric method, laser nephelometry and radial immunodiffusion [208]. Cell and tissue α2-M localization is also performed [209].

Determination of activity and concentration of cathepsin D inhibitors in bacterial and plant extracts may appear biased as also proteases with acidic pH optima can be present in the material [210]. These proteases, either sensitive or insensitive to the inhibitors, reduce the value of inhibitor activity - inhibit the activity (sensitive) and cause proteolytic inactivation (insensitive). The outcome depends upon the pH of the extraction fluid and temperature and time of extract storage. In the case of stable micromolecular inhibitors, proteases are inactivated thermally and by means of trichloroacetic or perchloric acid. Trichloroacetic acid is removed from the supernatant, whereas perchloric acid is precipitated by means of potassium hydrogen and inhibitor activity is determined as above. The outcome can also be affected by the order in which reagents are added: inhibitor - enzyme - substrate or inhibitor - substrate - enzyme

[211]. The activity and concentration of inhibitors in the plasma and systemic fluids are calculated per unit of volume, in cell culture per cell count and in solid tissues per 1g or 1 μg DNA.

Results of immunohistochemical assessment of inhibitor distribution (mainly α2-M) in cells and tissues have been presented in color microphotographs. Reaction intensity is evaluated semiquantitatively with the scale: lack of reaction (-), weak positive reaction (±), positive reaction (+), strong positive reaction (++) [212, 213]. Cathepsin D is localized in tissues by means of biotin- and biman-labeled pepstatin [214, 215].

Conclusions

The use of inhibitors has broadened the knowledge of the structure of the catalytic site, the mechanism of action and contribution of cathepsin D to protein degradation in lysosomes. Attempts have been also made to apply the inhibitors in the therapy of diseases occurring with cathepsin D involvement. Especially cathepsin D inhibitors, including statin, play a role, due to their low toxicity. The application range is even wider as statin is also the basic component of certain inhibitors of HIV protease [216-222] and of many renin inhibitors used for the treatment of hypertension and atherosclerosis [108, 223-226].

Acknowledgements: The work was partially funded from scientific grant of Medical University of Bialystok no.

References

[1] Davies DR. The structure and function of aspartic proteinases. *Ann Rev Biophys Chem.* 1990;19:189-215.
[2] Ersmark K, Feierberg I, Bjelie S, Hamelink E, Hackett F, Blackman MJ, Hulten J, Samuelsson B, Aqvist J, Hallberg A. Potent inhibitors of the Plasmoolium falciparum enzymes plasmepsin I and II deroid of cathepsin D inhibitory activity. *J Med Chem*. 2004;47:110-122.
[3] Hill J, Phylip LH. Bacterial aspartic proteinases. *FEBS Lett.* 1997;409:357-360.
[4] Rűchel R, DeBernardis F, Ray TL, Sullivan PA, Cole GT. Candida acid proteinase. *J Med Vet Mycol.* 1992;30:123-132.
[5] Rupova L, Keilova H. Isolation and some properties of an acid protease from Fasciola hepatica. *Z Parasitenkol*. 1979;61:83-91.
[6] Faust PL, Kornfeld S, Chirgwin JM. Cloning and sequence analysis of cDNA for human cathepsin D. *Proc Natl Acad Sci USA*. 1985;82:4910-4914.
[7] Hasilik A. The early and late processing of lysosomal enzymes: proteolysis and compartmentation. *Experientia*. 1992;48:130-151.
[8] Erickson AH, Conner GE, Blobel G. Biosynthesis of a lysosomal enzyme. Partial structure of two transient and functionally distinct NH2-terminal sequences in cathepsin D. *J Biol Chem.* 1981;256:11224-11231.
[9] Larsen LB, Boisen A, Petersen TE. Procathepsin D cannot antoactivation to cathepsin D at acid pH. *FEBS Lett.* 1993; 319:54-58.
[10] Richo GR, Conner GE. Structural requirements of procathepsin D activation and maturation. *J Biol Chem.* 1994;269: 14806-14812.
[11] Wittlin S, Rosel J, Hofman F, Stover DR. Mechanism and kinetics of procathepsin D activation. *Eur J Biochem.* 1999; 265:384-393.
[12] Yonezawa S, Takahashi T, Wang X, Wong RNS, Hartsuck JA, Tang J. Structure at the proteolytic processing region of cathepsin D. *J Biol Chem.* 1988;263:16504-16511.
[13] Lah T, Turk V. Autolysis studies of cathepsin D. Hoppe-Seyler's. *Z Physiol Chem.* 1982;363:247-254.
[14] Puizdar V, Turk V. Cathepsinogen D: characterization and activation to cathepsin D and inhibitory peptides. *FEBS Lett.* 1981;132:299-304.
[15] Samarel AM, Ferguson AG, Decker RS, Lesch M. Effects of cysteine protease inhibitors on rabbit cathepsin D maturation. *Am J Physiol.* 1989;257:C1069-C1079.
[16] Authier F, Mort JS, Bell AW, Posner BL, Bergeron JJM. Proteolysin of glucagons within hepatic endosomes by membrane-associated cathepsin B and D. *J Biol Chem.* 1995;270: 15798-15807.
[17] Diment S, Leech MS, Stahl PD. Cathepsin D in membrane-associated in macrophage endosomes. *J Biol Chem.* 1988; 263:6901-6907.
[18] Marciniszyn J, Hartsuck JA, Tang J. Mode of inhibition of acid proteases by pepstatin. *J Biol Chem.* 1976;251:7088-7094.
[19] Van Noort JM, Van der Drift ACM. The selectivity of cathepsin D suggests an involvement of the enzyme in the generation of T-cell epitopes. *J Biol Chem*. 1989;264:14159-14164.
[20] Ikeda K, Suzuki H, Okano T, Nakagawa S. Human spleen cathepsin D: its characterization and localization in human spllen. *Int J Biochem.* 1989;21:317-326.
[21] Saftig P, Hetman M, Schmal W, Weber K, Heine L, Mossmann H, Kőster A, Hess B, Evers M, von Figura K, Peters C. Mice deficient for the lysosomal proteinase cathepsin D exhibit progressive atrophy of the intestinal mucosa and profound destruction of lymphoid cells. *EMBO J.* 1995;14:3599-3608.
[22] Pagano M, Capony F, Rochefort H. In vitro activation of procathepsin B by cathepsin D both secreted by cancers cells. *C R Acod Sci Paris*, 1989;309:7-12.
[23] Van der Stappen JWJ, Williams AC, Maciewicz RA. Activation of cathepsin B, secreted by a colorectal cancer cell line requires low pH and is mediated by cathepsin *D. Int J Canc.* 1996;67:547-554.
[24] Wiederanders B, Kirschke H. The processing a cathepsin L precursor in vitro. *Arch Biochem Biophys.* 1989;272:516-521.
[25] Morris BJ. Activation of human inactive ("pro") rennin by cathepsin D and pepsin. *J Clin Endocrinol Metab.* 1978;46: 153-157.
[26] Lenarcic B, Kos J, Dolenc I, Lucovnik P, Križaj I, Turk V. Cathepsin D inactivates cysteine proteinase inhibitors cystatins. *Biochem Biophys Res Commun.* 1988;154:765-722.
[27] Lenarcic B, Krasovec M, Ritonja A, Olafsson I, Turk V. Inactivation of human cystatin C and kininogen by human cathepsin D. *FEBS Lett.* 1991;280:211-215.
[28] Kopitar M, Brzin J, Drobnic-Kosorek M, Babnik J, Locnikar P, Turk V, Giraldi T, Sava G. Some further characteristics of endogenous proteinase inhibitors. *Acta Biol Med Germ*. 1982;41:75-82.
[29] Drobnic-Kosorok M, Kopitar M, Babnik J, Turk V. Inactivation studies of the leucocyte inhibitor of urakinase by cathepsin D. *Mol Cell Biochem.* 1981;36:129-134.
[30] Sandhaus RA, Janoff A. Degradation of human α1-antytripsin by hepatocyte acid cathepsins. *Ann Rev Resp Dis.* 1974;110: 263-272.

[31] Pimenta DC, Chen VC, Chao J, Juliano MA, JulianoL. α1-antychymotrypsin and kallistatin hydrolysis by human cathepsin D. *J Protein Chem.* 200;19:411-418.
[32] Conover CA, Perry JE, Tindall DJ. Endogenous cathepsin D-mediated hydrolysis of insulin-like growth factor-binding proteins in cultured human prostatic carcinoma cells. *J Clin Endocrinol Metab.* 1995;80:987-992.
[33] Diment S, Martin KJ, Stahl PD. Cleavage of parathyroid hormone in macrophage endosomes illustrates a novel pathway for intracellular processin of proteins. *J Biol Chem.* 1989;264: 13403-13406.
[34] Hilfiker-Kleiner D, Kamiński K, Podewski E, Bonda T, Schaefer A. A cathepsin D-clewved 16 kDa from of prolactin mediater postpartum cardiomyopathy. *Cell.* 2007;128:589-600.
[35] Hook VYH, Azaryan AV, Hwang SR, Tezapsidis N. Proteases and emerging role of protease inhibitors in prohoruone processing. *FASEB J.* 1994;8:1269-1278.
[36] Kageyama T. Rabbit procathepsin E and cathepsin E. *Eur J Biochem*. 1993;216:717-728.
[37] Offermann MK, Chlebowski JF, Bond JS. Action of cathepsin D on fructose-1,6-bisphosphate aldolase. *Biochem J.* 1983; 211:529-534.
[38] Beaujouin M, Baghdiguian S, Glondu-Lassis M, Berchem G, Liaudet-Coopman E. Overexpression of both catalytically active and inactive cathepsin D by cancer cells enhances apoptosis-dependent chemo-sensivity. *Oncogene.* 2006;23: 1967-1973.
[39] Berchem G, Glondu M, Gleizes M, Brouillet JP, Vignon F, Garcia M, Liaudet-Coopman E. Cathepsin-D affects multiple tumor progression in vivo: proliferation, angiogenesis and apoptosis. *Oncogene.* 2002;21:5951-5955.
[40] Chwieralski CE, Welte T, Buhling F. Cathepsin-regulated apoptosis. *Apoptosis.* 2006;11:143-150.
[41] Davidson Y, Gibbons L, Pritchard A. Genetic associations Between cathepsin D exon 2 C→T polymorphism and Alzheimer's disease, and pathological correlations with genotype. *J Neurol Neurosurg Psychiat.* 2006;77:515-517.
[42] Fusek M, Vetvicka V. Dual role of cathepsin D: ligand and protease. *Biomed Papers.* 2005;149:43-50.
[43] Isidoro C. et al. Synthesis, maturation and extracellular release of procathepsin D as influenced by cell proliferation or transformation. *Int J Canc.* 1995;63:866-871.
[44] Jedinak A, Maliar T. Inhibitors of proteases as anticancer drugs. *Neoplasma.* 2005;52:185-192.
[45] Johanson AC, Steen H, Ollinger K, Roberg K. Cathepsin D mediates cytochrome C release and caspase activation in human fibroblast apoptosis induced by staurosporine. *Cell Deafh Different.* 2003;10:1253-1259.
[46] Laurent-Matha V, Maruani-Herrman S, Prebois C, Beaujoin M, Gondu M. Catalytically inactive human cathepsin D triggers fibroblast invasive growth. *J Cell Biol.* 2005;168:489-499.
[47] Leist M, Jäättelä M. Triggering of apoptosis by cathepsins. *Cell Death Different.* 2001;8:324-326.
[48] Liaudet-Coopman E, Beaujonin M, Derocq D, Garcia M, Glondu-Lassis M, Laurent-Matha V, Prebois C, Rochefort H, Vigon F. Cathepsin D: newly discovered functions of a long-standing aspartic protease in cancer and apoptosis. *Cancer Lett.* 2006;237:167-179.
[49] Minarowska A, Minarowski L, Karwowska A, Gacko M. Regulatory role of cathepsin D in apoptosis. *Folia Histochem Cytobiol.* 2007;45:159-163.
[50] Roberg K, Ollinger K. Oxidative stress causes relacation of the lysosomal enzyme cathepsin D with ensuing apoptosis in neonatal rat cardiomyocytes. *Am J Pathol.* 1998;152:1151-1156.
[51] Rochefort H, Liaudet E, Garcia M. Alternations and role of human cathepsin D in cancer metastasis. *Enzyme Protein.* 1996;49:106-116.
[52] Simon DI, Ezratty AM, Loscalzo J. The fibrin(ogen)olytic properties of cathepsin D. *Biochemistry.* 1994;33:6555-6563.
[53] Tian G, Solotka-Briners CD, Zysk J, Liu X, Birr C, Sylvester MA, Edwards PD, Scott CD, Greenberg BD. Linear non-competifive inhibition of solubilized human X-secretase by Pepstatin A methylester L685458, sulfonamides, and benzodiazepines. *J Biol Chem.* 2002;277:31499-31505.
[54] Devasagayam TPA, Pushpendran CK, Eapen J. Cyclohexiimide-induced inhibition of cathepsin D activity in suckling and adult rats. *Biochem Int.* 1981;3:55-60.
[55] Gordon PB, Seglen PO. Exogenous control of intracellular protein catabolism. In: *Proteolytic enzymes a practical approach*. Beynon RJ, Bond JS (eds). IRL Press Oxford. 1994:201-210.
[56] Turk V, Lah T, Puizdar V, Kregar I, Pain RH. The existence of a precursor of cathepsin D: evidence from autolysis, denaturation and activation studies. *Acta Biol Med Germ.* 1981;40: 1439-1450.
[57] Dionyssiou-Asteriou A, Rakitzis ET. Activarion of cathepsin D by glycine ethl ester. *Biochem J.* 1979;177:355-356.
[58] Watabe S, Ikeda T, Yago N. Activation of cathepsin D by polyanionic compounds. *Bioch Molec Biol Int.* 1996;39:703-710.
[59] Watabe S, Yago N. Phospholipids activate cathepsin D. *Biochem Biophys Res Commun.* 1983;110:934-939.
[60] Press EM, Porter RR, Cebra J. The isolation and properties of a proteolytic enzyme cathepsin D from bovine spleen. *Biochem J.* 1960;74:501-514.
[61] Kazakova OV, Orekhovich VN. Some properties of cathepsin chemically fixed to carriers. *Int J Pept Prot Res.* 1975; 7:23-29.
[62] Kimura H, Tsudzuki T, Murachi T. Proteolytic degradation of hemoglobin-haptoglobin compleks by lysosomal enzymes from rat liver. *J Biochem.* 1975;77:909-912.
[63] Antonov VK. *Chemistry of proteolysis*. Springer-Verlag Berlin. 1993;246-276.
[64] Bieth JG. Theoretical and practical aspects of proteinase inhibitors kinetics. *Meth Enzymol.* 1995;248:59-84.
[65] Davidson VL, Sittman DB. *Biochemistry.* Harwal Publ Philadelphia. 1994:90-91.
[66] Dixon M. The determination of enzyme inhibitor constans. *Biochem J.* 1953;55:170-171.
[67] Worowski K. Cell proteolysis and its regulation. *Post Biol Kom*. 1977;4:243-278.
[68] Ikezawa H, Aoyagi T, Takeuchi T, Umezawa H. Effect of protease inhibitors of actinomycetes on lysosomal peptide-hydrolases from swine liver. *J Antibiot.* 1971;14:488-490.
[69] Barrett AJ. Cathepsin D and other carboxyl proteinases. In: *Proteinases in mammalian cells and tissues*. Barrett AJ (ed). North-Holland Amsterdam. 1977:209-248.
[70] Fusek M, Mares M, Vagner J, Voburka Z, Baudys M. Inhibition of aspartic proteinases by propart peptides of human procathepsin D and chicken pepsinogen. *FEBS Lett.* 1991;287:160-162.
[71] Masa M, Maresova L, Vondrasek J, Horn M, Jezek J, Mares M. Cathepsin D propeptide: mechanism and regulation of its interaction with the catalytic core. *Biochemistry.* 2006;45:15474-15482.
[72] Starkey PM, Barrett AJ. α2-macroglobulin, a physiological regulator of proteinase activity. In: *Proteinases in mammalian cells and tissue*. Barrett AJ (ed). North-Holland Publ Comp Amsterdam. 1977:663-696.
[73] Shibata M, Koike M, Waguri S, Zhang G, Koga T, Uchiyama Y. Cathepsin D is specifically inhibited by deoxyribonucleic acids. *FEBS Lett*. 2002;517:281-284.
[74] Richardson M. The proteinase inhibitors of plants and microorganisms. *Phytochemistry.* 1977;16:159-169.

[75] Takahashi K, Chang WJ, Ko JS. Specific inhibition of acid proteases from brain, kidney skelet muscle, and insectiovorous plants by diazoacetyl-DL-norleucine methyl ester and by pepstatin. *J Biochem.* 1974;76:897-899.
[76] Worowski K. Cell inhibitors of proteolytic enzymes. *Post Biol Kom*. 1976;3:51-74.
[77] Xie D, Gulnik S, Collins L, Gustchina E, Suvorov L, Erickson JW. Dissection of the pH dependence of inhibitor binding energetics for an aspartic protease: direct measurement of the protonation states of the catalytic aspartic acid residues. *Biochemistry.* 1997;36:16166-16172.
[78] Smith GD, Murray MA, Nichols LW, Trikojus VM. Thyroid acid proteinase. Properties and inactivation by diazoacetyl - norleucine methyl ester. *Biochim Biophys Acta.* 1969;171: 288-298.
[79] Keilova H. Inhibition of cathepsin D by diazoacetylnorleucine methyl ester. *FEBS Lett.* 1970;6:312-314.
[80] Bayliss RS, Knowles JR, Wybrandt EB. An aspartic acid residue at the active site of pepsin. The isolation and sequence of the heptapeptide. *Biochem J.* 1969;113:377-386.
[81] Stepanov VM, Orekhovich VN, Lobareva LS, Mzhelskaya TI. Action of the pepsin inhibitor on the bovine spleen cathepsin D. *Biochemistry (USSR)*. 1969;34:170-171.
[82] Kaehn KK, Morr M, Kula MR. Inhibition of the acid proteinase from Neurospora crassa by diazoacetyl-DL-norleucine methyl ester, 1,2-epoxy-3-(4-nitrophenoxy) propane and pepstatin. *Z Physiol Chem.* 1979;360:791-794.
[83] Kregar I, Stanovnik B, Tisler M, Nisi C, Gubensek F, Turk V. Inactivation studies of cathepsin D with diazo compounds. *Acta Biol Med Germ*. 1977;36: 1927-1930.
[84] Takahashi K, Chang WJ. The structure and function of acid proteases. V. Comparative studies on the specific inhibition of acid proteases by diazoacetyl-DL-norleucine methyl ester, 1,2-epoxy-3-(p-nitrophenoxy) propane and pepstatin. *J Biochem*. 1976;80:497-506.
[85] Tang J. Evolution in the structure and function of carboxyl proteases. *Mol Cell Biochem.* 1979;26:93-109.
[86] Rao CM, Scarborough PE, Kay J, Batley B, Rapundalo S, Klutchko S, Taylor MD, Lunney EA, Humblet CC, Dunn BM. Specificity in the binding of inhibitors to the active site of human/primate aspartic proteinases: analysis of P2-P1-P1,-P2, wariations. *J Med Chem.* 1993;36:2614-2620.
[87] Rakitzis ET, Malliopoulou TB. Inactivation of cathepsin D by dithiophosgene and by 2,2-dichloro-1,3-dithiacyclobutanone. *Biochem J.* 1976;153:737-739.
[88] Keilova H, Markovic O, Keil B. Characterization and chemical modification of cathepsin D from bovine spleen. *Collect Czech Chem Commun.* 1969;34:2154-
[89] Keilowa H, Keil B. Specificity of cleavage of heptapeptide substrate with bovine spleen cathepsin D. *Collect Czech Chem Commun.* 1968;33:131-140.
[90] Tang J. Specific and irreversible inactivation of pepsin by substrate - like epoxides. *J Biol Chem.* 1971;246:4510-4517.
[91] Keilova H. On the specificity and inhibition of cathepsin D and B. In: *Tissue proteinases.* Barrett AJ, Diugle JT. North-Holland Publ Co Amsterdam. 1971:45-67.
[92] Lin TY, Williams HR. inhibition of cathepsin D by synthetic oligopeptides. *J Biol Chem.* 1979;254:11875-11883.
[93] Ikezawa H, Yamada K, Aoyagi T, Takeuchi T, Umezawa H. Effect of antipain on lysosomal peptide hydrolases from swine liver. *J Antibiot.* 1972;25:738-740.
[94] Aoyagi T, Morishima H, Nishizawa R, KunimotoS, Takeuchi T. Biological activity of pepstatins, pepstatone A and partial peptides on pepsin, cathepsin D and renin. *J Antibiot.* 1972; 25:689-694.
[95] Doi E, Shibata D, Matoba T, Yonezawa D. Characterization of pepstatin-sensitive acid protease in resting rice seeds. *Agric Biol Chem.* 1980;44:741-747.
[96] Gunn JM, Owens RA, Liu WS, Glover GJ. Biological activity of aspartic proteinases inhibitors related to pepstatin. *Acta Biol Med Germ.* 1981;40:1547-1553.
[97] Jupp RA, Dunn BM, Jacobs JW, Vlasuk G, Arcuri KE, Veber DF, Perlow DS, Payne LS, Boger J, Laszlo S, Chakravarty PK, Broekes J, Hangauer DG, Ondeyka D, Greenlee WJ, Kay J. The selectivity of statine-based inhibitors against various human aspartic proteinases. *Biochem J.* 1990;265:871-878.
[98] Woessnr JF. Pepstatin inhibits the digestion of hemoglobin and protein-polisaccharide complex by cathepsin D. *Biochem Biophys Res Commun.* 1972;47:965-970.
[99] Evin G, Sharples RA, Weidman A, Reinhard FBM, Carbone V, Culvenor JG, Holsinger RM, Sernee MF, Beyreuter K, Masters CL. Aspartyl protease inhibitor pepstatin binds to the presenilins of Alzheimers disease. *Biochemistry.* 2001;40: 8359-8368.
[100] Wingender W. Proteinase inhibitors of microbial origin. In: Proteinase inhibitors. Fritz H, Tschesche H, Greene LJ, Truscheit E (eds). Springer Verlag. 1974:548-559.
[101] Umezawa H. Protease inhibitors produced by microorganisms. *Acta Biol Med Germ.* 1977;36:1899-1915.
[102] Baldwin ET, Bhat TN, Gulnik S, Hosur MV, Soder I, Cachau RE, Collin J, Silva AM, Erickson JW. Crystal structures of native and inhibited forms of human cathepsin D: implications for lysosomal targeting and drug design. *Proc Natl Acad Sci USA.* 1993;90:6796-6800.
[103] Cooper JB. Aspartic proteinases indisease: a structural perspective. *Curr Drug Targets.* 2002;3:155-173.
[104] Rich D. Pepstatin - derived inhibitors of aspartic proteinases. A close look at an apparent transitionstate analog inhibitor. *J Med Chem.* 1985;28:263-273.
[105] Majer P, Collins JR, Gulnik SV, Erickson JW. Structure-based subside specificity mapping of human cthepsin D using statine-based inhibitors. *Protein Sci.* 1997;6:1458-1466.
[106] Hasilik A, Figura K, Conzelman E, Nehrkorn H, Sandhoff K. Lysosomal enzyme precursors in human fibroblasts. Activation of cathepsin D precursor in vitro and activity of beta-hexosaminidase A precursor toward ganglioside GM2. *Eur J Biochem.* 1982;125:317-321.
[107] Agarwal NS, Rich DH. Inhibition of cathepsin D by substrate analognes containing statine and by analognes of pepstatin. *J Med Chem.* 1986;29:2519-2524.
[108] Boger J, Lober NS, Ulm EH. Novel renin inhibitors containing the amino acid statine. Nature. 1983;303:81-84.
[109] Sarubbi E, Seneci PE, Angelastro MR, Peet NP, Denarto M, Islam K. Peptide aldehydes as inhibitors of HIV protease. *FEBS Lett.* 1993;319:253-256.
[110] Wood JM, Gulati N, Forgiarini P, Fuhver W, Hofbauer KG. Effects of a specific and long-acting rennin inhibitor in the marmoset. *Hypertension.* 1985;7:797-803.
[111] Tone H, Matsushita Y, Yagi Y, Takamatsu A, Aoyagi T, Takeuchi T, Umezawa H. Purification and properties of pepstatin hydrolase from Bacillus sphaericus. *J Antibiot.* 1975;28:1012-1015.
[112] Matsushita Y, Tone H, Hori S, Yagi Y, Takamatsa A, Horishima H, Aoyagi T, Tekeuchi T, Umezawa H. N-acylated derivatives of a peptide obtained by enzymatic degradation of pepstatins. *J Antibiol.* 1975;28:1016-1018.
[113] Dumas J, Brittelli D, Chen J, Dixon B, Hatoum-Mokdad H. Synthesis and structure activity relationships of novel small molecule cathepsin D inhibitors. *Bioorg Med Chem Lett.* 1999;9:2531-2536.
[114] Huo S, Wang J, Cieplak P, Kollman PA, Kuntz ID. Molecular dynamics and free energy analysis of cathepsin D-inhibitor interactions: insight into structure-based ligand design. *J Med Chem.* 2002;45:1412-1419.

[115] Kick EK, Roe DC, Skillman AG, Liu G, Ewing TJ, Sun Y, Kuntz ID, Ellman JA. Structural-based design and combinatorial chemistry yield low molecular inhibitors of cathepsin D. *Chem Biol.* 1997;4:297-307.
[116] Lee CE, Kick EK, Ellman JA. General solid-phase synthesis approach to prepare mechanism-based aspartyl protease inhibitor libraries. Identification of potent cathepsin D inhibitors. *J Am Chem Soc.* 1998;120:9735-9747.
[117] Farley PC, Christeller JT, Sullivan ME, Sullivan PA, Laing WA. Analysis of the interaction between the aspartic peptidase inhibitor SQAPI and aspartic peptidases using surface plasmon resonance. *J Mol Recognit.* 2002; 15:135-144.
[118] Koiwa H, Bressan RA, Hasegawa PM. Regulation of protease inhibitors and plant defense. *Trends Plant Sci.* 1997;2: 379-384.
[119] Mutlu A, Gal S. plant aspartic proteinases: enzymes ou the way to a function. *Physiol Plant.* 1999;105:569-576.
[120] Ryan CA. Protease inhibitors in plants: genes for improving defenses against insects and pathogens. *Ann Rev Phytopathol.* 1990;28:425-449.
[121] Taylor BH, Young RJ, Scheuring CF. Induction of a protease inhibitor II-class gene by aucin in tomato roots. *Plant Molecul Biol.* 1993;23:1005-1014.
[122] Baudys M, Ghosh M, Harlos K, Meres M, Fusek M, Kostka V, Blake CC. Crystallization and preliminary crystallographic study of cathepsin D inhibitor from potatoes. *J Mol Biol.* 1991;218:21-22.
[123] Hannapel DJ. Nucleotide and deduced amino acid sequence of the 22-kilodalton cathepsin D inhibitor protein of potato (Solanum tuberosum L.). *Plant Physiol.* 1993;101:703-704.
[124] Herbers K, Prat S, Willmitzer L. Cloning and characterization of a cathepsin D inhibitor gene from Solanum tuberosum L. *Plant Molec Biol.* 1994;26:73-83.
[125] Keilova H, Tomasek V. Isolation and some properties of cathepsin D inhibitor from potatoes. *Collect Czech Chem Commun.* 1975;41:489-497.
[126] Keilova H, Tomasek V. Naturally occurring inhibitors of intracellular proteinases. *Acta Biol Med Germ.* 1977;36: 1873-1881.
[127] Maganja DB, Strukelj B, Pungercar J, Gubensek F, Turk V, Kregar I. Isolation and sequence analysis of the genomic DNA fragment encoding and aspartic proteinase inhibitor homologue from potato (Solanum tuberosum L.). *Plant Mol Biol.* 1992;20:311-313.
[128] Mares M, Fusek M, Kostka V, Baudys M. Cathepsin D inhibitor from potato tubers (Solanum tuberosum L.). *Adv Exp Med Biol.* 1991;306:349-353.
[129] Mares M, Meloun B, pavlik M, Kostka V, Baudys M. primary structure of cathepsin D inhibitor from potatoes and its structure relationship to soybean trypsin inhibitor family. *FEBS Lett.* 1989;251:94-98.
[130] Ritonja A, Krizaj I, Mesko P, Kopitar M, Lucovnik P, Strukelj B, Pungeracar J, Buttle DJ, Barrett AJ, Turk V. The amino acid sequence of a novel inhibitor of cathepsin D from potato. *FEBS Lett.* 1990;267:13-15.
[131] Strukelj B, Pungercar J, Ritonja A, Krizaj I, Gubensek F, Kregar I, Turk V. Nucleotide and deduced amino acid sequence of an aspartic proteinase inhibitor homologue from potato tubers (Solanum tuberosum L.). *Nucleic Acids Res*. 1990;18:4605.
[132] Dash C, Kulkarni A, Dunn B, Rao M. Aspartic peptidase inhibitors: implications in drug development. *Crit Rev Biochem Molec Biol.* 2003;38:89-119.
[133] Ishikawa A, Ohta S, Matsuoka K, Hattori T, Nakamura K. A family of potato genes that encode Kunitz - type proteinase inhibitors i structural comparisons and differential expression. *Plant Cell Physiol.* 1994;35:303-312.
[134] Guevara MG, Daleo GR, Oliva CR. Purification and characterieation of an aspartic protease from potato leaves. *Physiol Plantorum.* 2001;112:321-326.
[135] Christeller JT, Farley PS, Ramsay RJ, Sullivan PA, Laing WA. Purification, characterization and cloning of an aspartic proteinase inhibitor from squash phloem exudates. *Eur J Biochem.* 1998;254:160-167.
[136] Mucha D. The recognition of plant derived inhibitors of cathepsin D. M.B.S. Thesis (Polish), Pharmaceutical Faculty, Medical University of Bialystok, 1997.
[137] Galleschi L, Friggeri M, Repiccioli R, Come D. Aspartic proteinase inhibitor from wheat: some properties. In: *Proceedings of Fourth Int. Workshop Seads: basic and applied aspects of seed biology.* Corbineau F. Angers France. 1993:207-211.
[138] Sarkkinen P, Kalkkinen N, Tilgmann C, Siuro J, Kervinen J, Mikola L. Aspartic proteinase from barlej grains is related to mammalian lysosomal cathepsin D. *Planta*.1992;186:317-323.
[139] Cleveland TE, Black LL. Partial purification of proteinase inhibitors from tomato pants infected with Phytophtora infestans. *Phytopathology.* 1983;73:664-670.
[140] Lison P, Rodrigo I, Conejero V. A novel function for the cathepsin D inhibitor in tomato. *Plant Physiol.* 2006;142: 1329-1339.
[141] Werner R, Guitton MC, Muhlbach HP. Nucleotiole sequence of a cathepsin-D inhibitor protein from tomato. *Plant Physiol.* 1993;103:1473.
[142] Balandin T, Van der Does C, Belles AJM, Bol JF, Linthorst HJM. Structure and induction pattern of a novel proteinase inhibitor class II gene of tobacco. *Plant Mol Biol*. 1995;27: 1197-1204.
[143] Cater SA, Lees WE, Hill J, Kay J, Phylip LH. Aspartic proteinase inhibitors from tomato and potato are more potent against yeast proteinase A than cathepsin D. *Biochim Biophys Acta.* 2002;596:76-82.
[144] Hansen JD, Hannapel DJ. A wound-inducible potato proteinase inhibitor gene expressed in non-tuber-bearing species is not sucrose inducible. *Plant Physiol.* 1992;100: 164-169.
[145] Farmer EE, Johnson RR, Ryan CA. Regulation of expression of proteinase inhibitor genes by methyl jasmonate and jasmonic acid. *Plant Physiol.* 1992;98:995-1002.
[146] Ishikawa A, Yoshihara T, Nakamura K. Jasmonate-inducible expression of a potato cathepsin D inhibitor - GUS gene fusion in tobacco cells. *Plant Mol Biol.* 1994;26:403-414.
[147] Kreft S, Ravnikar M, Mesko P, Pungercar J, Umek A, Kregar I, Strukelj B. Jasmonic acid inducible aspartic proteinase inhibitors from potato. *Phytochemistry.* 1997;44:1001-1006.
[148] Pena-Cortes H, Sanchez-SerranoJ, Mertena R, Willmitzer L, Part S. Abscisic acid is involved in the wound-induced expression of the proteinasese inhibitor II gene in potato and tomato. *Proc Natl Acad Sci USA.* 1989;86:9851-9855.
[149] Strukelj B, Ravnikar M, Mesko P, Poljsak-Prijatelj M, Pungercar J, Kopitar G, Kregar I, Turk V. Molecular cloning and immunocytochemical localization of jasmonic acid inducible cathepsin D inhibitor from potato. *Adv Exp Med Biol.* 1995;362:293-298.
[150] Doares SH, Narvaez-Vasques J, Conconi A, Ryan CA. Salicylic acid inhibits synthesis of proteinase inhibitors in tomato leaves induced by systemin and jasmonic acid. *Plant Physiol.* 1995;108:1741-1745.
[151] Brunelle F, Cloutier C, Michaud D. Colorado potato beetles compensate for tomato cathepsin D inhibitor expressed in transgenic potato. *Arch Insect Biochem Physiol.* 2004;55:103-113.
[152] Rickauer M, Fournier J, Esquerre-Tugaye MT. Induction of proteinase inhibitors in tobacco cell suspension culture by

elicitors of Phytophthora parasitia var. nicotianae. *Plant Physiol.* 1989;90:1065-1070.
[153] Galesa K, pain R, Jongsma MA, Turk V, Lenarcic B. Structural characterization of thyreoglobulin type-1 domains of equistatin. *FEBS Lett.* 2003;539:120-124.
[154] Lenarcic B, Turk V. Tyreoglobulin type-1 domains in equistatin inhibit both papain-like cystein proteinases and cathepsin D. *J Biol Chem*. 1999;274:563-566.
[155] Martzen MR, McMullen BA, Smith NE, Fujikawa K, Peansky RJ. Primary structure of the major pepsin inhibitor from the internal parasitic nematode Ascaris suum. *Biochemistry.* 1990;29:7366-7372.
[156] Ng KKS, Petersen MM, Charney MM, Garen C, Zalatoris JJ, Rao-Naik C, Dunn BM, Martzen MR, Peansky RJ, James MNG. Structural Basis for the inhibition of porcine pepsin by Ascaris pepsin inhibitor-3. *Nat Struct Biol.* 2000;7:653-657.
[157] Valler MJ, Kay J, Aoyagi T, Dunn BM. The interaction of aspartic proteinases with naturally-occurring inhibitors from actinomycetes and Ascaris lumbricoides. *J Enzyme Inhib.* 1985;1:77-82.
[158] Wiederanders B, Kirschke H. The processing a cathepsin L precursor in vitro. *Arch Biochem Biophys.* 1989;272:516-521.
[159] Kageyama T. Molecular cloning, expression and characterization of an Ascaris inhibitor for pepsin and cathepsin E. *Eur J Biochem.* 1998;253:804-809.
[160] Roberts RC, Hall PK. Specificity of proteinases for the "bait" region of α1-macroglobulin. *Ann NY Acad Sci.* 1983;421:61-68.
[161] Travis J, Salvesen GS. Human plasma proteinase inhibitors. *Ann Rev Biochem.* 1983;52:655-709.
[162] Sottrup-Jensen L, Londlad PB, Stepnik TM, Petersen TE, Magnuson S, Jornvall H. Primary structure of the 'bait' rgion for proteinases in α2-macroglobulin. *FEBS Lett.* 1981;127:167-173.
[163] Bodmer JL, Schnebli HP. Plasma proteinase inhibitors. *Schweiz Med Wschr.* 1984;114:1359-1363.
[164] Lah T, Vihar M, Turk V. Interaction of cathepsin D and pepsin with α2-macroglobulin. In: *Aspartic proteinases and their inhibitors*. Kostka V. Walter de Gruyter Berlin. 1985:485-490.
[165] Sttrup-Jensen L. α-macroglobulins: structure, shape, and mechanism of proteinase complex formation. *J Biol Chem.* 1989;264:11539-11542.
[166] Thomas DJ, Richards AD, Kay J. Inhibition of aspartic proteinases by α2-macroglobulin. *Biochem J.* 1989;259:905-907.
[167] Gaspar A, Skosey JL, Sequeira W, Teodorescu M. Detection of α2-macroglobulin-associated proteases in the plasma of patients with rheumatoid arthritis. *Clin Chem.* 1984;30:1517-1522.
[168] Gonias SL, Pizzo SV. Conformation and protease binding activity of binary and ternary human α2-macroglobulin-protease complex. *J Biol Chem.* 1983;258:14682-14692.
[169] Barrett AJ, Starkey PM. The interaction of α2-macroglobulin with proteinases. Characteristics and specificity of the reaction, and hypothesis concerning its molecular mechanism. *Biochem J.* 1973;133:709-724.
[170] Blatrix C, Amouch P, Drouet J, Steinbuch M. Study on the plasmatic elimination of the α2-macroglobulin proteinase complexes. *Path Biol.* 1973;21:11-14.
[171] Witt I, Tritschler W, Bablok W. α2-macroglobulin: reference values in serum and plasma with a chromogenic substrate (chromozym® TRX). *J Clin Chem Clin Biochem*. 1981;19:877-878.
[172] Kaplan J, Nielsen ML. Analysis of macrophage surface receptors 1. Binding of α2-macroglobulin-protease complex to rabbit alveolar macrophages. *J Biol Chem.* 1979;254:7323-7328.
[173] Swenson RP, Howard JB. Characterization of alkylamine-sensitive site in α2-macroglobulin. *Proc Natl Acad Sci USA.* 1979;7614:4313-4316.
[174] Avila JL, Convit J. Inhibition of leucocytic lysosomal enzymes by glicosaminoglycans in vitro. *Biochem J.* 1975;152:57-64.
[175] Weston PD, Poole AR. Antibodies to enzymes and their uses, with specific reference to cathepsin D and other lysosomal enzymes. In: *Lysosomes in biology and pathology*. Dingle JT (ed). North Holland Elsevier Amsterdam. 1973;3:425-464.
[176] Dingle JT, Barrett AJ, Weston PD. Cathepsin D. Characteristics of immunoinhibition and the confirmation of a role in cartilage breakdown. *Biochem J.* 1971;123:1-13.
[177] Kopitar-Jerala N, Puizdar V, Berbic S, Zavasnik-Bergant T, Turk V. A cathepsin D specific monoclonal antibody. *Immunol Lett.* 2001;77:125-126.
[178] Weston PD. A specific antiserum to lylosomal cathepsin D. *Immunology.* 1969;17:421-428.
[179] Dingle JT, Poolo R, Lazarus GS, Barrett AJ. Immunoinhibition of intracellular protein digestion in macrophages. *J Exp Med.* 1973;137:1124-1141.
[180] Tulkens P, Trouet A, Van Hoof F. Immunological inhibition of lysosome function. *Nature.* 1970;228:1282-1285.
[181] Hoffmann RHJ, Ristow J, Veser J, Frank W. properties of two growth stimulating proteins isolated from fetal calf serum. *Exp Cell Res.* 1973;80:275-280.
[182] Yonezawa H, Uchikoba T, Kaneda M. Determination of pepstatin-sensitive carboxyl proteases by using pepstatinyldansyldiaminopropane (dansyl-pepstatin) as an active site titrant. *J Biochem.* 1997;122:294-299.
[183] Afting EG, Becker ML. Two-step affinity-chromatgraphic purification of cathepsin D from myometrium with high yield. *Biochem J.* 1981;197:519-522.
[184] Gubensek F, Barstow I, Kregar I, Turk V. Rapid isolation of cathepsin D by affinity chromatography on the immobilized synthetic inhibitor. *FEBS Lett.* 1976;71:42-44.
[185] Huang JS, Huang SS, Tang J. Cathepsin D isozymes from porcine spleens. Large scale purification and polypeptide chain arrangements. *J Biol Chem.* 1979;254:11405-11417.
[186] Kregar I, Urh I, Umezawa H, Turk V. Purification of cathepsin D by affinity chromatography on pepstatin-Sepharose resin. *Croat Chim Acta.* 1977;49:587-592.
[187] Conner GE. Isolation of procathepsin D from mature cathepsin D by pepstatin affinity chromatography. Autocatalytic proteolysis of the zymogen from of the enzyme. *Biochem J.* 1989;263:601-604.
[188] Stewart AJ, Piggott NH, May FEB, Westley BR. Mitogenic activity of procathepsin D purified for conditioned medium of breast cancer cells by affinity chromatography on pepstatinyl agarose. *Int J Cancer.* 1994;57:715-718.
[189] Strukelj B, Pungercar J, Mesko P, Barlic-Maganja D, Gubensek F, Kregar I, Turk V. Characterisation of aspartic proteinase inhibitors from potato at the gene, cDNA and protein levels. *Biol Chem Hoppe Seyler.* 1992;373:477-482.
[190] Wittlin S, Rösel J, Stover DR. One-step purification of cathepsin D by affinity chromatography using immobilized propeptide sequences. *Eur J Biochem.* 1998;252:530-536.
[191] Huisman W, Lanting L, Doddema HJ, Bouma JMW, Gruber M. Role of individual cathepsins in lysosomal protein digestion as tested by specific inhibitors. *Biochim Bioplys Acta*. 1974;370:297-307.
[192] Kominami E, Ueno T, Muno D, Katunuma N. The selective role of cathepsin B and D in the lysosomal degradation of endogenous and exogenous proteins. *FEBS Lett.* 1991;287:189-192.

[193] Weston PD, Barrett AJ, Dingle JT. Specific inhibition of cartilage breakdown. *Nature.* 1969;222:285-286.
[194] Yamato S, Hirabayashi Y, Sigihara H. an improved procedure for the histochemical demonstration of cathepsin D by the mercury-labeled pepstatin method. *Stain Technol.* 1984; 59:113-120.
[195] Yamamoto K, Kamata O, Katsuda N, Kato K. Immunochemical difference between cathepsin D and cathepsin E-like enzyme from rat spleen. *J Biochem.* 1980;87:511-516.
[196] Greenbaum LM. Sutherland JHR. Host cathepsin D response to tumor in the normal and pepstatin-treated mouse. *Cancer Res.* 1983;43:2584-2587.
[197] Iodice AA, Leong V, Weinstock JM. The inhibition by pepstatin of cathepsin D and autolysis of dystrophic muscle. *Arch Biochem Biophys.* 1966;117:477-486.
[198] Lin TY, Williams HR. Inhibition of the local hemorrhagic Shwartzman reaction by an acid proteinase inhibitor, pepstatin. *Experientia.* 1975;31:209-212.
[199] Takashima T, Kawada N, Maeda N, Okuyama H, Uyama N, Seki S, Arakawa T. Pepstatin A attenuates the inhibitory effect of N-acetyl-L-cysteina on proliferation of hepatic myofibroblast (stellate cells). *Eur J Pharmacol.* 2002;451: 265-270.
[200] Tumminello FM, Bernacki RJ, Gebbia N, Leto G. Pepstatins: aspartic proteinase inhibitors having potential therapeutic applications. *Med Res Rev.* 1993;13:199-208.
[201] Umezawa H, Aoyagi T. Activities of proteinase inhibitors of micro bal origin. In: *Proteinases in mammalian cell and tissues*. Barret AJ (ed). North-Holland Publ Comp Amsterdam. 1977:637-662.
[202] Jones RCW, Wangensteen SI. Protective effect of cathepsin D antiserum on hemorrhagic shock. *Surg Forum.* 1975;26: 29-31.
[203] Salvesen G, Nagase H. Inhibition of proteolytic enzymes. In: *Proteolytic enzymes a practical approach*. Beynon RJ, Bond JS (eds). IRL Press Oxford. 1994:83-104.
[204] Cheng Y, Prusoff WH. Relationship between the inhibition constant (Ki) and the concentration of inhibitor whid causes 50 percent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol.* 1973;22:3099-3108.
[205] Knight CG. Principles of the design and use of synthetic substrates and inhibitors for tissue proteinases. In: *Proteinases in mammalian cells and tissues*. Barrett AJ. North-Holland Amsterdam. 1977:583-636.
[206] Umezawa H. Structure and activities of protease inhibitors of microbial origin. *Meth Enzymol.* 1976;45:678-695.
[207] Knight CG. Active site titration of peptidases. *Meth Enzymol.* 1995;248:85-101.
[208] Barrett AJ. α2-macroglobulin. *Meth Enzymol.* 1981;80:737-754.
[209] Cassiman JJ, Van Leuven F, Van der Schueren B, Van den Borghe H. Immunohistochemical localization of human alpha2-macroglobulin in connective tissue. *Cell Tissue Res.* 1980;213:301-310.
[210] Sznajd J, Naskalski J. The assessment of activity of inactivated enzyme during catalytical process. *Diagn Lab.* 1978; 14:127-133.
[211] Chi CW, Lo SS, Tan FL, Zhang YS, Chu HM. Studies on the mung bean trypsin inhibitor. In: *Proteins in biology and medicine*. Bradshaw RA, Hill RL, Tang J (eds). Acad Press NY. 1982:341-362.
[212] Reid WA, Valler MJ, Kay J. Immunolocalisation of cathepsin D in normal and neoplastic human tissues. *J Clin Pathol.* 1986;39:1323-1330.
[213] Rochefort H, Liaudet E, Garcia M. Alternations and role of human cathepsin D in cancer metastasis. *Enzyme Protein.* 1996;49:106-116.
[214] Matthews ITW, Decker RS, Knight CG. The localization of cathepsin D with a biotin-labeled pepstatin. *FEBS Lett.* 1981;134:253-255.
[215] Matthews ITW, Decker RS, Knight CG. Bimane-labeled pepstatin, a fluorescent probe for the subcellular location of cathepsin D. *Biochem J.* 1981;199:611-617.
[216] Alteri E, Bold G, Cozensr. CGP 53437, an orally bioavailable inhibitor of human immunodeficiency virus type 1 protease with potent antiviral activity. *Antimicrob Agents Chemother.* 1993;37:2087-2092.
[217] Le VD, Mak CC, Lin YC, Elder JH, Wong CH. Structure-activity studies of FIV and HIV protease inhibitors containing allophenylnorstatine. *Bioorg Med Chem.* 2001;9:1185-1195.
[218] Leung D, Abbenate G, Fairlie DP. Protease inhibitors: current ststus and future prospects. *J Med Chem.* 2000;43:305-341.
[219] Ogden RC, Flexner CW. *Protease inhibitors in AIDS therapy*. Marcel Dekker NY. 2001.
[220] Patick AK, Potts KE. Protease inhibitors as antiviral agents. *Clin Microbiol Rev*. 1998;11:614-627.
[221] Von der Helm K, Gűrtler L, Eberle J, Deinhardt F. Inhibition of HIV replication in cell culture by the specific aspartic protease inhibitor pepstatin A. *FEBS Lett.* 1989;247:349-352.
[222] Wlodawer A, Erickson JW. Structure based inhibitors of HJV-1 protease. *Ann Rev Biochem.* 1993;62:543-585.
[223] Januszewicz W, Januszewicz A, Prejbisz A. Renin inhibitors (Inhibitory reniny). *Nefrologia Nadciśnienie Tętnicze.* 2006;6:9-14.
[224] Jones DM, Sueiras-Diaz J, Szelke M, Leckie BJ, Beattie SR, Morton J, Neidle S, Kuroda R. New rennin inhibitors containing novel analogues of statine. *J Pept Res.* 1997;50:109-121.
[225] Rich DH. Inhibitors of aspartic proteinases. In: *Proteinase inhibitors*. Barrett A.J, Salvesen G. Elsevier Amsterdam. 1986:179-217.
[226] Rosenson RS, Tangney CC. Antiatherothrombotic properties of statins. Implication for cardiovascular event reduction. *JAMA*. 1998;279:1643-1650.

Submitted: 14 September, 2007
Accepted after reviews: 16 October, 2007