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Mechanism-Based Isocoumarin Inhibitors for Serine Proteases: Use of Active Site Structure and Substrate Specificity in Inhibitor Design

James C. Powers, Chih-Min Kam, Lakshmi Narasimhan, Joszef Oleksyszyn, Maria A. Hernandez, and Toshihisa Ueda

School of Chemistry, Georgia Institute of Technology, Atlanta, Georgia 30332

Isocoumarins are potent mechanism-based heterocyclic irreversible inhibitors for a variety of serine proteases. Most serine proteases are inhibited by the general serine protease inhibitor 3,4-dichloroisocoumarin, whereas isocoumarins containing hydrophobic 7-acylamino groups are potent inhibitors for human leukocyte elastase and those containing 7-alkylureidogroups are inhibitors for porcine pancreatic elastase. Isocoumarins containing basic side chains that resemble arginine are potent inhibitors for trypsin-like enzymes. A number of 3-alkoxy-4-chloro-7-guanidinoisocoumarins are potent inhibitors of bovine thrombin, human factor Xa, human factor XIa, human factor XIIa, human plasma kallikrein, porcine pancreatic kallikrein, and bovine trypsin. Another cathionic derivative, 4-chloro-3-(2-isothiureidoethoxy) isocoumarin, is less reactive toward many of these enzymes but is an extremely potent inhibitor of human plasma kallikrein. Several guanidinoisocoumarins have been tested as anticoagulants in human plasma and are effective at prolonging the prothrombin time. The mechanism of inhibition by this class of heterocyclic inactivators involves formation of an acyl enzyme by reaction of the active site serine with the isocoumarin carbonyl group. Isocoumarins with 7-amino or 7-guanidino groups will then decompose further to quinone imine methide intermediates, which react further with an active site residue (probably His-57) to form stable inhibited enzyme derivatives. Isocoumarins should be useful in further investigations of the physiological function of serine proteases and may have future therapeutic utility for the treatment of emphysema and coagulation disorders.

Key words: anticoagulants, blood coagulation enzymes, elastase, emphysema, isocoumarins, molecular modeling

Serine proteases are involved in a wide variety of important physiological processes, including blood clotting, fibrinolysis (dissolution of clots), reproduction, kinin and hormone processing, the regulation of blood pressure, the immune defense,

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phagocytosis (destruction of foreign cells and particles), and tissue turnover [1]. Normally the activity of these enzymes is carefully controlled by very effective, specific high-molecular-weight plasma inhibitors. If the natural inhibitors are absent or defective, destruction of normal tissue and disease can result.

In pulmonary emphysema, there is firm link between the disease and serine proteases. Emphysema is caused by the destruction to the elastic lung protein elastin by the enzymes elastase and cathepsin G [2]. Over 35,000 Americans die from this disease each year and many more are disabled. The elastase that causes most of the lung damage is normally present in human neutrophils, is essential for phagocytosis, and is only destructive when released into the plasma and not controlled by its natural plasma protein inhibitor (α_1-protease inhibitor).

Intravascular clotting is another major health problem in the United States that involves serine proteases. Venous thromboembolism annually causes approximately 300,000 patients to be hospitalized and more than 50,000 deaths. About 948,000 people annually suffer an acute myocardial infarction (227,000 deaths), and coronary thrombosis is present in the majority of infarcts. Anticoagulant, antithrombotic, and hemostatic drugs are therefore important tools in the arsenal of the physician. The coagulation pathway is now fairly well understood, and most of the coagulation factors are serine proteases.

Other disease states that involve serine proteases are inflammation and arthritis (neutrophil elastase, neutrophil cathepsin G, mast cell chymase, mast cell tryptase); blistering (mast cell chymase and tryptase); autoimmune diseases and organ rejection (complement proteases, natural killer cell proteases, and T-lymphocyte proteases); viral infections (host and viral processing serine proteases); and pancreatitis (pancreatic chymotrypsin, trypsin, and elastase).

ACTIVE SITE STRUCTURE AND MOLECULAR MODELING

Molecular modeling is one technology that is rapidly improving and becoming useful to the biochemist for the process of inhibitor design. A necessary starting point is an x-ray crystal structure of the target protease or a close relative. With mammalian serine proteases, x-ray structures are now available for several chymotrypsin-like enzymes (bovine pancreatic α- and γ-chymotrypsin, and rat mast cell protease II), trypsin-like enzymes (bovine trypsin and porcine pancreatic kallikrein), and elastases (porcine pancreatic elastase and human neutrophil elastase). In addition, an increasing number of small-molecule inhibitor complexes with serine proteases are becoming available.

The three-dimensional structures of serine proteases are surprisingly similar, especially in their active-site regions. The geometric orientation of the catalytic residues (Ser-195, His-57, Asp-102) is highly conserved in various serine proteases, although the binding of substrates and inhibitors will often cause movements of these residues. The peptide backbones of active-site residues that interact with substrates also have similar conformations in serine proteases whose x-ray structures have been determined, although human leukocyte (HL, human neutrophil) elastase and rat mast cell protease II (RMCP II) have nearby insertions or deletions of amino acid residues or short peptide fragments that cause small changes in the backbone conformations. However, the major changes in both primary and secondary specificity of serine

proteases are brought about by changes in amino acid side chains in the substrate binding region of the active site.

The primary specificity pocket (S_1), in particular, is a major determinate of serine protease specificity and is most often utilized in inhibitor design. This pocket is a bumpy hydrophobic space in most serine proteases, with a variety of amino acid side chains forming the surface of the pocket (Fig. 1). The primary substrate binding subsite takes on a different size and shape in individual serine proteases because of sequence differences in this region of serine proteases. For example, the presence of Asp-189 in trypsin-like enzymes is responsible for their cleavage of substrates following lysine and/or arginine residues, whereas Thr-226 and Val-216 fill this space in PP elastase, resulting in a preference for substrates with small P_1 side chains, such as alanine, valine, and serine.

The two neutrophil proteases (HL elastase and cathepsin G) have a long, narrow S_1 pocket with carboxyl groups at the very end. In the case of HL elastase, Asp-226 points toward the interior of the protein into a region that has several water molecules trapped in the elastase structure [3]. The front of the pocket is very narrow and hydrophobic. A similar situation occurs with cathepsin G, where residue 226 is a glutamic acid residue. Construction of a cathepsin G model using the RMCP II backbone indicates that this Glu-226 would be placed in a similar environment to Asp-226 in HL elastase.

Previous studies with synthetic peptide thiobenzyl esters [4] have shown that cathepsin G and RMCP II prefer substrates with a P_1 Phe, while HL and porcine pancreatic (PP) elastase most effectively hydrolyzed a norvaline containing substrate (Table I). Interestingly, inhibitors with long hydrocarbon or fluorocarbon chains that can interact with this hydrophobic S_1 pocket are often the most effective HL elastase inhibitors.

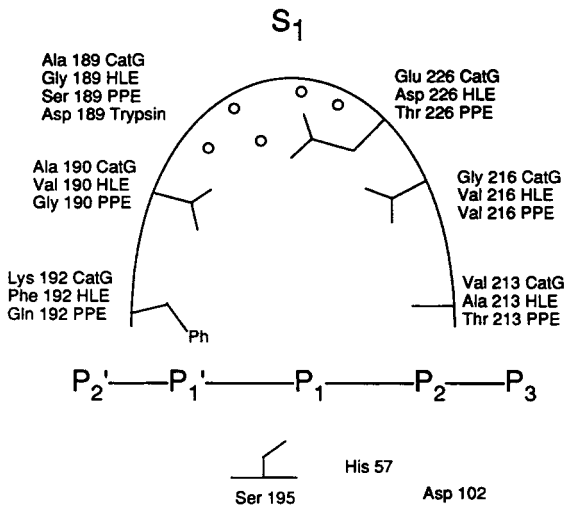


Fig. 1. Schematic drawing of the primary substrate binding Site (S_1) Of HL elastase. The corresponding residues for cathepsin G (CatG), porcine pancreatic elastase (PPE), and trypsin are shown, along with those of HL elastase (HLE). The side chain of Asp-226 is shown pointing toward the back of the pocket into a region that contains several water molecules.

TABLE I. Substrate Specificity of Mammalian Serine Proteases: Specificity Constant (k_{cat}/K_M) for the Hydrolysis of Boc-Ala-Ala-AA-SBzl*

	k_{cat}/K_M ($M^{-1}s^{-1}$) Amino acid residue (AA)				
Protease	Ala	Val	Nva	Leu	Phe
HLE[a]	730,000	3,000,000	9,200,000	2,200,000	No Rxn
PPE	740,000	660,000	6,800,000	4,400,000	No Rxn
CatG	No Rxn	No Rxn	210,000	220,000	1,000,000
RMCP II	No Rxn	No Rxn	740,000	220,000	2,100,000

*Hepes buffer (0.1 M), pH 7.5, 0.5 M NaCl, and 10% Me_2SO at 25°C.
[a]Abbreviations used: HLE, human leukocyte elastase; PPE, porcine pancreatic elastase; CatG, human cathepsin G; RMCP II, rat mast cell protease II.

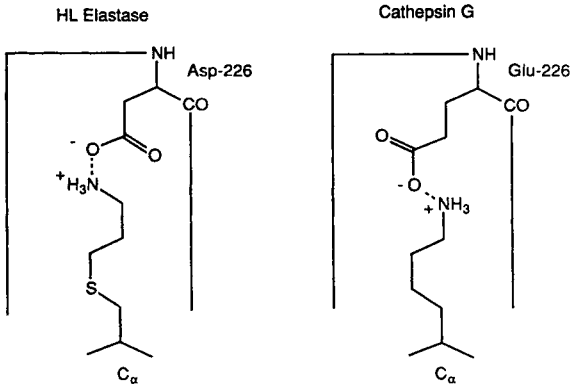


Fig. 2. Schematic drawing of the active sites of HL elastase interacting with a substrate containing a S-(3-aminopropyl)cysteine residue and cathepsin G interacting with a lysine substrate. The S-(3-aminopropyl)cysteine residue is one atom longer than a lysine residue and can be considered thiohomolysine.

The presence of Asp-226 in HL elastase and Glu-226 in cathepsin G suggested the possibility that these neutrophil enzymes might hydrolyze peptide substrates containing cationic side chains in the P_1 position. We therefore synthesized MeO-Suc-Ala-Ala-Pro-Lys-SBzl and MeO-Suc-Ala-Ala-Pro-Apc-SBzl [Apc = S–(3–aminopropyl) cysteine; $H_2NCH_2CH_2CH_2SCH_2CH(NH_2)CO_2H$]. Modeling indicated that the S-(3-aminopropyl) cysteine side chain would fit best into the narrow S_1 pocket of HLE and could reach Asp-226, although some close contacts would be formed. In contrast, the lysine derivative appeared to be more appropriate for the cathepsin G primary substrate binding site S_1 (Fig. 2).

Kinetic data with the two substrates indicated that the two cationic substrates were indeed hydrolyzed by HL elastase, with the S-(3-aminopropyl) cysteine derivative being 19-fold more effective (Table II). However, both were much less reactive than the valine substrate MeO-Suc-Ala-Ala-Pro-Val-SBzl. The low hydrolysis rates probably indicate that the cationic side chains are not going into the S_1 subsite completely, but may loop in and then out of S_1, with the ammonium group hydrogen bonding with a surface residue. In the case of cathepsin G, the Lys substrate was equally reactive with the excellent cathepsin G substrate Suc-Ala-Ala-Pro-Phe-SBzl. However, the K_M value was about tenfold higher, which may indicate weaker binding; the k_{cat} value was tenfold higher. The S-(3-aminopropyl) cysteine substrate has a

TABLE II. Specificity of Neutrophil Serine Proteases for Cationic Substrates: Kinetic Constants for the Hydrolysis of MeO-Suc-Ala-Ala-Pro-AA-SBzl*

Amino acid residue (AA)	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}s^{-1}$)
		Cathepsin G	
Phe[a]	0.048	14	300,000
Phe[b]	0.019	22	1,200,000
Lys	0.26	170	650,000
Apc[c]	0.21	360	1,700,000
		Human neutrophil elastase	
Val	0.0023	13	5,600,000
Lys			3,500
Apc			67,000
		Porcine pancreatic elastase	
Lys	0.092	0.73	7,900
Apc			14,000
		Bovine trypsin	
Lys	0.058	130	2,300,000
Apc	0.0084	10	1,200,000

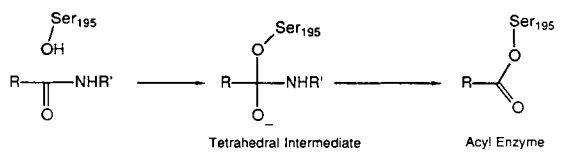
*Hepes buffer (0.1 M), pH 7.5, 0.5 M NaCl or 0.01 M $CaCl_2$, and 10% Me_2SO at 25°C.
[a]The substrate used was Suc-Ala-Ala-Pro-Phe-SBzl.
[b]The substrate used was Suc-Val-Pro-Phe-SBzl.
[c]Apc, S-(3-aminopropyl) cysteine.

similar K_M value, compared with the Lys derivative, however its k_{cat} value was about twofold higher and the resulting k_{cat}/K_M value was threefold higher. This substrate is slightly more reactive than the previous best thioester substrate for cathepsin G, Suc-Val-Pro-Phe-SBzl [5]. It remains to be seen whether inhibitors can be designed to make use of the cationic interaction in the S_1 site of either HLE or cathepsin G and whether there is any physiological relevance to the hydrolysis of substrates with basic side chains.

INHIBITOR DESIGN

Synthetic serine protease inhibitors [see 6 for a recent review] have great potential for use in the treatment of a variety of diseases. As a result, there is considerable activity in the design of new types of inhibitors, especially for enzymes such as human leukocyte (neutrophil) elastase. Surprisingly, much less attention is given to the design of inhibitors for blood coagulation serine proteases, and essentially no new anticoagulant drugs have appeared in recent years.

Mechanism-based and transition state analogs are two types of serine protease inhibitors that are being actively investigated by a number of laboratories as possible therapeutic agents. The mechanism of serine protease action involves formation of a tetrahedral intermediate by reaction of Ser-195 with the carbonyl group of the peptide substrate. This is followed by generation of an acyl enzyme.



Most transition state analog protease inhibitors such as peptide trifluoromethyl ketones and boronic acids give enzyme-inhibitor complexes that resemble the tetrahedral intermediate. Mechanism-based inactivators (or suicide inhibitors) require enzyme catalysis to release a latent reactive group contained in the inhibitor structure, before inactivation can occur. Most of these inhibitors require initial formation of an acyl enzyme structure, which then undergoes further reaction to either stabilize the acyl enzyme or unmask additional reactive groups.

Isocoumarins are one class of heterocyclic structure that is rich in possible masked functional groups. At present, isocoumarins containing latent acid chloride (or ketenes) or quinone imine methide functional groups have been investigated as serine protease inhibitors (Fig. 3). Reaction first occurs between the isocoumarin ring carbonyl with the active site serine (Ser-195) of a serine protease to form an acyl-enzyme, which is often quite stable. With the appropriately substituted isocoumarin rings, further reaction can then release other latent functional groups in the active site of the enzyme for reaction with another side chain of the enzyme.

DICHLOROISOCOUMARIN-A GENERAL SERINE PROTEASE INHIBITOR

Dichloroisocoumarin is a mechanism-based inhibitor that reacts with all serine proteases tested so far [7]. Serine proteases that are inhibited include elastases (porcine pancreatic and human neutrophil); chymotrypsin-like enzymes (bovine chymotrypsin, cathepsin G, rat mast cell protease I, rat mast cell protease II, skin chymase, natural killer cell chymase); trypsin-like enzymes (skin tryptase, lung tryptase, bovine trypsin, plasmin, pancreatic kallikrein, plasma kallikrein, natural killer cell tryptase); coagulation enzymes (thrombin, factor Xa, factor XIa, factor XIIa, and factor VIIa); complement enzymes (protein D, C1s̄, and factor I), and

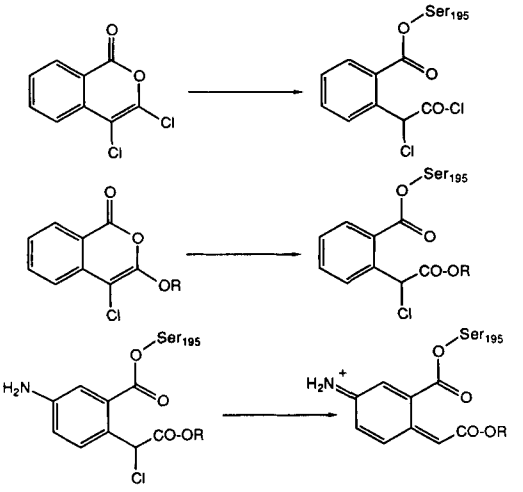


Fig. 3. Reactive groups released upon formation of acyl enzyme derivatives during reaction of serine proteases with isocoumarin inhibitors. The **top** structure shows 3,4-dichloroisocoumarin reacting to form an acid chloride, or ketene, in the active site. In the **middle**, a 3-alkoxy-4-chloroisocoumarin is reacting to form a stable acyl enzyme. At the **bottom**, a 3-alkoxy-7-amino-4-chloroisocoumarin has reacted to form a quinone imine methide structure.

protease La. Subtilisin reacts with 3,4-dichloroisocoumarin but appears to turn over the inhibitor.

The mechanism of the inhibition reaction involves acylation of the active-site serine by 3,4-dichloroisocoumarin with the release of an acid chloride (or ketene) in the active site. Enzyme-catalyzed isocoumarin ring opening occurs concurrently with inactivation. Proton-release studies suggest that 3,4-dichloroisocoumarin forms either a diacylated enzyme structure upon inactivation of chymotrypsin or a monoacylated enzyme in which the acid chloride has been hydrolyzed (Fig. 4). Such an acyl enzyme could be stabilized by a salt link between the carboxylate of the inhibitor and the protonated His-57.

The rates of inhibition of serine protease by 3,4-dichloroisocoumarin vary from $k_{obsd}/[I] = 8900\ M^{-1}s^{-1}$ for human leukocyte elastase to 10–200 $M^{-1}s^{-1}$ for most trypsin-like enzyme. At effective 3,4-dichloroisocoumarin concentrations of 50–400 μM, most serine protease are inhibited with $t_{1/2}$ in the range of 0.3 to 5 min. The acyl enzymes formed have varying stabilities, with half-lives for reactivation >24 h when excess inhibitor is not removed and about 6–10 h when inhibitor is removed by dialysis. However, the acyl enzymes can be reactivated quickly by the addition of hydroxylamine. Dichloroisocoumarin is an easily handled crystalline material, is stable for months in organic solvents, is reasonably stable in buffer, and does not react with other classes of enzymes [$k_{obsd}/[I] = 0.5\ M^{-1}s^{-1}$ with acetyl cholinesterase; no inhibition with papain, β-lactamase, and leucine aminopeptidase]. These properties make it a useful alternate to the more toxic diisopropylphosphofluoridate (DFP), and it is generally much more reactive toward serine proteases than phenylmethanesulfonyl fluoride (PMSF).

Dichloroisocoumarin may also be useful for characterization of the role of serine proteases in biological processes. It has recently been shown to be an effective inhibitor of lymphocyte granule-mediated cytolysis of target cells [8]. The granules contain both chymase and tryptase activity, which are inactivated by a number of other serine protease inhibitors, but 3,4-dichloroisocoumarin was effective in abrogating the physiological function of the granules.

3-ALKOXY AND 3-ALKOXY-7-AMINOISOCOUMARIN SERINE PROTEASE INHIBITORS

Simple 3-alkoxy-4-chloroisocoumarins and 3-alkoxy-4-chloro-7-nitroisocoumarins are potent acylating agents for a variety of serine proteases, including elastases and chymotrypsin-like enzymes [9,10]. In the acyl enzyme, an ester function group is formed for the 3-alkoxy group, and no further reaction with the enzyme occurs since

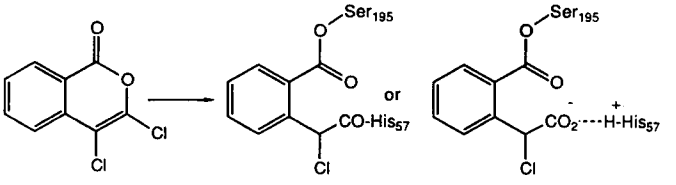


Fig. 4. Mechanism of inhibition of serine proteases by 3,4-dichloroisocoumarin. The inhibited enzyme could be either a doubly acylated derivative (**center**) or an acyl enzyme in which a salt link stabilizes the acyl derivative (**right**).

the acyl enzymes can readily be reactivated with hydroxylamine. In many cases the acyl enzymes are quite stable.

The most reactive 3-alkoxy-4-chloroisocoumarins have $k_{obsd}/[I]$ values as high as 46,000 $M^{-1}s^{-1}$. The reactivity toward a particular serine protease depends on the structure of the 3-alkoxy group. For instance, HL elastase is inactivated most rapidly by 4-chloro-3-ethoxyisocoumarin ($k_{obsd}/[I]$ = 43,000 $M^{-1}s^{-1}$) and reacts with the 3-benzyloxy derivative about 30-fold more slowly. Similarly, chymotrypsin-like enzymes react most rapidly with the 3-benzyloxy derivatives ($k_{obsd}/[I]$ = 46,000 $M^{-1}s^{-1}$) for rat mast cell protease I with 4-chloro-3-(4-fluorobenzyl)isocoumarin. Because this reactivity matches the primary specificity of the various serine proteases, the results suggested that the 3-alkoxy group is interacting with the S_1 subsite of the enzyme.

Addition of a 7-amino group to the 3-alkoxy-4-chloroisocoumarin ring yields another class of isocoumarins (3-alkoxy-7-amino-4-chloroisocoumarins) that are potent inactivators of elastase and chymases [9,10]. Again, the inactivation rates (Table III) depended on the structure of the 3-alkoxy substituent, which strongly indicates that this substituent interacts with the S_1 subsite of the enzyme. For example, one of the most reactive HL elastase inhibitors, 7-amino-4-chloro-3-methoxyisocoumarin ($k_{obsd}/[I]$ = 10,000 $M^{-1}s^{-1}$), has a relatively small alkyl substituent, whereas the best rat mast cell protease I inhibitor ($k_{obsd}/[I]$ = 910 $M^{-1}s^{-1}$) has a bulky aromatic substituent [3-(2-phenylethyl)].

In addition to being reactive, these 7-amino-4-chloroisocoumarins are also efficient inactivators, often having small partition ratios (ratio of inhibitor turnover/enzyme inactivation). Enzymatic ring opening is required for inhibition, and the inactivated enzymes are extremely stable to reactivation after removal of excess inhibitor. For example, HL and PP elastase inactivated by 7-amino-3-ethoxy-4-chloroisocoumarin regained no detectable activity upon standing after dialysis.

The available evidence points to the inactivation mechanism shown in Figure 5. Enzyme acylation results in the formation of an acyl enzyme that can undergo an elimination reaction to produce a 4-quinone imine methide (Fig. 5, left) in the active site. This can react either with an enzyme nucleophile (probably His-57) to give an irreversibly inhibited enzyme structure (Fig. 5, top right) or with a solvent nucleophile to give a stable acyl enzyme (Fig. 5, bottom right). Partial reactivation (42% at pH 7.5 with PP elastase) by hydroxylamine suggests a partitioning between the two

TABLE III. Inhibition of Mammalian Serine Proteases by 3-Alkoxy-7-Amino-4-Chloroisocoumarins*

Serine protease	$k_{obsd}/[I]$ ($M^{-1}s^{-1}$) 3-Alkoxy substituent		
	Methoxy	Ethoxy	Phenylethoxy
HLE	10,000	9,400	40
PPE	1,000	700	No Rxn
CatG	17	200	70
ChyT[a]	110	270	1,200
RMCP I	80	590	910
RMCP II	130	880	30

*Hepes buffer (0.1 M), pH 7.6, 0.6 M NaCl, and 10% Me_2SO at 25°C.
[a]*Abbreviations used: ChyT, bovine chymotrypsin; RMCP, rat mast cell protease I.*

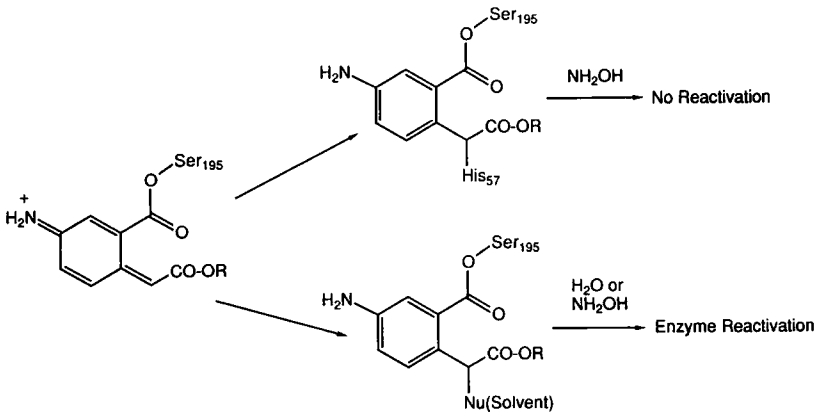


Fig. 5. Mechanism of inhibition of serine proteases by 3-alkoxy-7-amino-4-chloroisocoumarins.

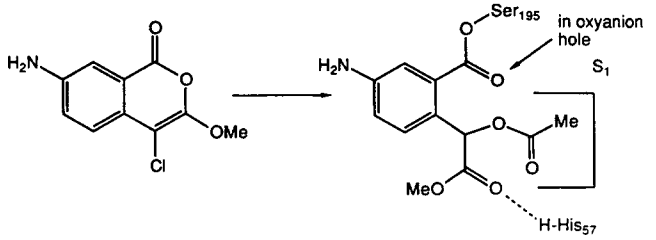


Fig. 6. Schematic drawing of the complex of porcine pancreatic elastase with 7-amino-4-chloro-3-methoxyisocoumarin.

enzyme-inhibitor complexes, with 58% being the nonreactivatable complex probably containing an alkylated histidine residue. Both the 7-amino and 4-chloro groups are required for formation of a stable inactivated enzyme; and isocoumarins that lack these features inhibit serine proteases but deacylate fairly rapidly [9].

The crystal structures of two complexes of isocoumarins with porcine pancreatic elastase have been solved to atomic resolution. PP elastase inactivated by 7-amino-4-chloro-3-methoxyisocoumarin has been solved at pH 5 in 0.1 M acetate buffer to a resolution of 0.18 mm [11]. A single ester linkage is formed between the inhibitor and the active-site serine residue, whereas an acetate solvent molecule has added stereospecifically to the quinone imine methide intermediate or has directly displaced the chlorine atom in the acyl enzyme. A hydrogen bond exists between the imidazole ring of His-57 and the carbomethoxy group of the covalently bound inhibitor molecule (Fig. 6). The acetate moiety occupies the S_1 subsite. This inactivated elastase molecule can be reactivated by the addition of hydroxylamine, although slowly [9].

More recently, a crystal structure of PP elastase inhibited by 4-chloro-3-ethoxy-7-guanidinoisocoumarin at pH 5.0 has been completed (Meyer EF Jr, Radhakrishnan R: personal communication). Again, an acyl enzyme is formed, but in contrast to the 7-amino derivative, the alkoxy group is located in the S_1 subsite of the enzyme and the chlorine is still present (Fig. 7). The guanidino group is hydrogen bonded to the carbonyl group and side-chain hydroxyl of Thr-41. It is remarkable that the inhibitor moieties in the two complexes are rotated and translated with respect to each other,

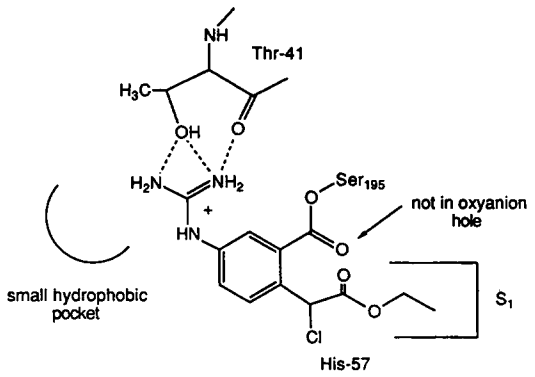


Fig. 7. Schematic drawing of the structure of PP elastase inhibited by 7-guanidino-4-chloro-3-ethoxyisocoumarin at pH 5.0.

TABLE IV. Inhibition of Porcine Pancreatic Elastase by 7-Substituted 3-Ethoxy-4-Chloroisocoumarins*

7-Substituent	$k_{obsd}/[I]$ ($M^{-1}s^{-1}$)
H_2N-	710
H_2N-C(=NH2$^+$)-NH-	2,300
H_2N-CO-NH-	2,200
Me-NH-CO-NH-	1,400
Et-NH-CO-NH-	1,700
i-Pr-NH-CO-NH-	4,900
t-Bu-NH-CO-NH-	8,100
C_6H_5-NH-CO-NH-	4,200

*Hepes buffer (0.1 M), pH 7.5, 0.5 M NaCl, and 10% Me_2SO at 25°C.

indicating that small heterocyclic inhibitors can bind in a variety of modes to serine proteases.

Examination of the environment of the 7-guanidino group in the PP elastase complex indicated the presence of a small hydrophobic region composed of portions of the hydroxyphenyl ring of Tyr-35, Cys-58, the hydrocarbon chain of Arg-61, one methyl group of Leu-63, and an edge of Phe-65. Molecular modeling indicated that small alkyl groups such as t-butyl could interact with this site. Alkyl carbamyl derivatives of 7-amino-4-chloro-3-ethoxyisocoumarin were synthesized; the inhibition results are shown in Table IV. Replacement of the guanidino group by a ureido group (NH_2-CO-NH-) resulted in no decrease in the inhibition rate, as might be expected given that the ureido isocoumarin can form the same number of hydrogen bonds with the enzyme. As predicted by molecular modeling, the best inhibitors were the t-butylcarbamyl and the phenylcarbamyl derivatives.

In the structure of human neutrophil elastase, Phe-41 has replaced Thr-41 of PPE, and some backbone movements have resulted from other nearby side-chain substitutions. Substitutions of large aromatic groups on the 7-amino group of the isocoumarin have resulted in some effective inhibitor structures for human neutrophil elastase (Table V), with the Tos-Phe derivative the most effective. At present, there are no crystal structures of isocoumarin inhibitors bound to HL elastase, but molecu-

TABLE V. Inhibition of Human Leukocyte Elastase by 7-Substituted 3-Methoxy-4-Chloroisocoumarins*

7-Substituent	$k_{obsd}/[I]$ ($M^{-1}s^{-1}$)
CH_3CO-HN-	30,000
$PhCH_2CO$-NH-	37,000
Ph_2CHCO-NH-	52,000
$PhCH_2CH_2CO$-NH-	66,000
Ph_2CHCH_2CO-NH-	130,000
Tos-Phe-NH-	200,000[a]

*Hepes buffer (0.1 M), pH 7.5, 0.5 M NaCl, and 10% Me_2SO at 25°C.
[a]Progress curve method at [S] = 193 μM.

lar modeling indicates that the phenylalanine side chain of the Tos-Phe derivative could make hydrophobic contacts with Phe-192 and Leu-143, whereas the tosyl group could interact with portions of Leu-35, Phe-41, and Val-62 of HL elastase. Other binding modes are possible, and further evidence will be necessary to confirm this model.

INHIBITORS FOR COAGULATION ENZYMES

Trypsin, porcine pancreatic kallikrein, and several blood-coagulation enzymes, including bovine thrombin, bovine factor Xa, human factor Xa, human plasma factor XIa, human plasma factor XIIa, human plasma kallikrein, human complement protein D, human $C1\bar{s}$, and capillary-injury-related protease (CIP), are inactivated by substituted isocoumarins containing basic functional groups (aminoalkoxy, guanidino, and isothiureidoalkoxy). The basic side chains provide recognition in the active sites of the blood-coagulation enzymes, which all bind and cleave at arginine residues in their natural peptide substrates. Figure 8 shows some representative inhibitor structures and $k_{obsd}/[I]$ values with bovine pancreatic trypsin [12,13]. It is interesting to note that placement of a wide variety of cationic groups in two different locations on the isocoumarin ring results, in effective inhibitors.

The most potent inhibitors for coagulation enzymes are 3-alkoxy-4-chloro-7-guanidinoisocoumarins, which have $k_{obsd}/[I]$ values in the range of 10^3–10^5 $M^{-1}s^{-1}$ (Table VI). The isothiureido derivative 4-chloro-3-isothiureidoalkoxyisocoumarins showed high inhibitory potency toward porcine pancreatic kallikrein, human plasma kallikrein, human factor XIa, human factor XIIa, and trypsin, with $k_{obsd}/[I]$ values of the order of 10^4–10^5 $M^{-1}s^{-1}$. The inhibition of these serine proteases by the substituted isocoumarins was time dependent, and the inactivation of trypsin by 3-alkoxy-4-chloro-7-guanidinoisocoumarins and 7-amino-4-chloro-3-(3-isothiureidopropoxy) isocoumarin occurred concurrently with the loss of the isocoumarin absorbance.

The mechanism of inhibition of 3-alkoxy-4-chloro-7-guanidinoisocoumarins is shown in Figure 9. Initially, an acyl enzyme is formed (top right), which probably can decompose further through a quinone imine methide (middle). The quinone imine methide can either react with solvent to give another acyl enzyme derivative (bottom right) or react with an active-site nucleophile such as His-57. This would yield another acyl enzyme derivative (bottom left), which would be quite stable toward reactivation. The complexes formed from inactivation of trypsin by 3-alkoxy-4-chloro-7-guanidi-

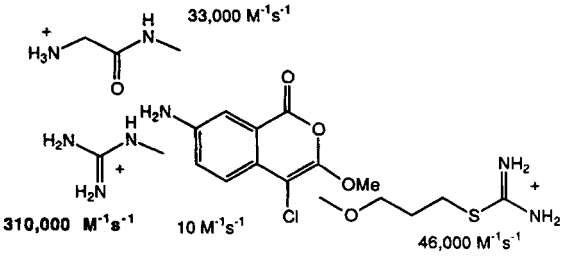


Fig. 8. Structures of isocoumarin inhibitors that contain cationic functional groups with $k_{obsd}/[I]$ values shown for the inhibition of bovine trypsin.

TABLE VI. Inhibition of Mammalian Trypsin-Like Serine Proteases by 3-Alkoxy-4-Chloro-7-Guanidinoisocoumarins*

Serine protease	$k_{obsd}/[I]$ ($M^{-1}s^{-1}$) 3-alkoxy substituent		
	Methoxy	Ethoxy	Phenylethoxy
B[a] Thrombin	290,000	55,000	30,000
B Factor Xa	3,100	27,000	96,000
H Factor Xa	11,000	11,000	11,000
H FActor XIa	36,000	60,000	20,000
H FActor XIIa	20,000	22,000	26,000
PP Kallikrein	45,000	200,000	200,000
HP Kallikrein	240,000	>500,000	>270,000
B Trypsin	310,000	>110,000	>110,000
H Protein D	250	190	90
H $C1\bar{s}$	660	690	90
CIP	620	2,200	3,900

*Hepes buffer (0.1 M), 0.01 M $CaCl_2$ or 0.5 M NaCl, pH 7.5, 8–10% Me_2SO at 25°C.
[a]Abbreviations used: B, bovine; H, human; PP, porcine pancreatic; HP, human plasma; CIP, capillary injury related protease.

noisocoumarins and 7-amino-4-chloro-3-(3-isothiureidopropoxy)isocoumarin were very stable and regained less than 4% of activity in Hepes buffer (pH 7.5) after 1 day at 25°C. Even treatment with buffered 0.29 M hydroxylamine resulted in the recovery of only 8–45% activity. Trypsin inactivated by 3-alkoxy-7-guanidinoisocoumarins with no halogen regained full activity upon standing or addition of hydroxylamine. Thrombin inactivated by 3-alkoxy-4-chloro-7-guanidinoisocoumarins was also stable and only regained 9–15% activity. The reactivation results support the mechanism (Fig. 9) that involves the formation of additional covalent bonds between the inhibitor and the enzyme after the initial acyl enzyme is formed.

Two inhibitors, 4-chloro-7-guanidino-3-methoxyisocoumarin and 4-chloro-3-ethoxy-7-guanidinoisocoumarin, are effective anticoagulants in human plasma. They prolonged the prothrombin time from 12 s to >2.8 min. Interestingly, the 3-phenylethoxyderivative was ineffective. Both active inhibitors had a short half-life in plasma and can be used only in applications where it is desirable to remove excess inhibitor quickly.

CONCLUDING REMARKS

It is clear that small-molecular-weight heterocyclic molecules can be very effective inhibitors for serine proteases. However, it is currently difficult to introduce

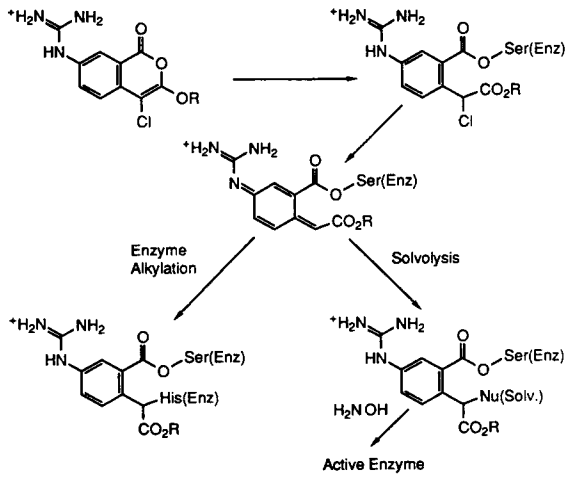


Fig. 9. Mechanism of inhibition of trypsin-like serine proteases by 4-chloro-3-ethoxy-7-guanidinoisocoumarin.

absolute specificity into small-molecule nonpeptide inhibitors. This is especially true for the family of trypsin-like enzymes, which contains many members with similar active-site structures and primary substrate specificity. Many of the isocoumarins discovered so far are general inhibitors and react with a number of serine proteases, although introduction of cationic groups into the inhibitor structure will usually distinguish trypsin-like enzymes from other serine proteases. It is important to note that small changes in the inhibitor structure can have large effects on the binding modes of the inhibitors, as determined by x-ray crystallography.

Prediction of the binding mode of novel heterocyclic inactivators with serine proteases is not yet practical with molecular modeling, although a fairly accurate prediction of the binding mode can be made with simple peptides. With serine proteases whose structures have been solved by x-ray crystallography, molecular modeling can give new insights and lead to the design of novel structures. Once an enzyme-inhibitor complex has been determined by x-ray crystallography, it can be extremely valuable for designing new inhibitors with improved potency. Models of serine proteases whose x-ray structures are not yet available can also be valuable tools in inhibitor design, as long as the models are approached with a healthy degree of skepticism and an exact fitting of inhibitor structures is not attempted. In the future, as our understanding of the binding of heterocyclic molecules to serine proteases improves, it will be possible to use molecular modeling to design and synthesize new inhibitor structures with improved specificity and efficacy for therapeutic use in the treatment of disease.

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REFERENCES

1. Neurath H: J Cell Biochem 32:35–49, 1986.
2. Powers JC, Bengali ZH: Am Rev Respir Dis 134:1097–1100, 1986.
3. Bode W, Wei AZ, Huber R, Meyer J, Travis J, Neumann S: EMBO J 5:2453–2458, 1986.
4. Harper JW, Cook RR, Roberts CJ, McLaughlin BJ, Powers JC: Biochemistry 23:2995–3002, 1984.
5. Powers JC, Tanaka T, Harper JW, Minematsu Y, Barker L, Lincoln D, Crumley KV, Fraki JE, Schechter NM, Lazarus GG, Nakajima K, Nakashino K, Neurath H, Woodbury RG: Biochemistry 24:2048–2058, 1984.
6. Powers JC, Harper JW: In Barrett AJ, Salvensen GS (eds): "Proteinase Inhibitors." Amsterdam, New York: Elsevier Science Publishers, 1986, pp 55–152.
7. Harper JW, Hemmi K, Powers JC: Biochemistry 24:1831–1841, 1985.
8. Hudig D, Gregg NJ, Kam CH, Powers JC: Biochem Biophys Res Commun 149:882–888, 1987.
9. Harper JW, Powers JC: Biochemistry 24:7200–7213, 1985.
10. Harper JW, Powers JC: J Am Chem Soc 106:7618–7619, 1984.
11. Meyer Jr EF, Presta LG, Radhakrishnan R: J Am Chem Soc 107:4091–4093, 1987.
12. Kam CM, Copher JC, Powers JC: J Am Chem Soc 109:5044–5045, 1987.
13. Kam CM, Fujikawa K, Powers JC: Biochemistry 27: 2547–2557, 1988.