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INHIBITION OF THROMBIN BY ARGININE–CONTAINING PEPTIDE CHLOROMETHYL KETONES AND BIS CHLOROMETHYL KETONE-ALBUMIN CONJUGATES

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Arg-containing peptide chloromethyl ketones including D-Phe-Pro-Arg-CH_2Cl derivatives have been synthesized and tested as inhibitors for thrombin and several blood coagulation enzymes. The parent compound, D-Phe-Pro-Arg-CH_2Cl is still the best thrombin inhibitor in the series with $k_{obs}/[I]$ value of 10^7 $M^{-1}s^{-1}$. Extension by one amino acid (Phe or Gly), or a peptide moiety ($ClCH_2$-Arg<-Pro<-D-Phe<-CO-CO-, $ClCH_2$-Arg<-Pro<-D-Phe<-CO-$(CH_2)_3$-CO-, where < – indicates a reversed amino acid residue, -CO-CHR-NH-) on the N-terminus of D-Phe-Pro-Arg-CH_2Cl reduces the inhibition constant by 1–2 orders of magnitude, which indicates the importance of a free amino group at the N-terminus. The tripeptide D-Phe-Pro-Arg-CH_2Cl and related tetrapeptide inhibitors inhibit thrombin more potently than factor IXa and plasma kallikrein by 2–5 orders of magnitude. Z-Arg-CH_2Cl and Phe-Phe-Arg-CH_2Cl which contain a large hydrophobic group at the P_2 site inhibit thrombin poorly. All the peptide chloromethyl ketones inhibit plasma kallikrein moderately with $k_{obs}/[I]$ values of 10^2–10^3 $M^{-1}s^{-1}$ but inhibit factor IXa poorly ($k_{obs}/[I]$ < 20 $M^{-1}s^{-1}$). Conjugates of albumin with the bis chloromethyl ketones [$(CO\text{-}D\text{-}Phe\text{-}Pro\text{-}Arg\text{-}CH_2Cl)_2$, $(CH_2)_3$-$(CO\text{-}D\text{-}Phe\text{-}Pro\text{-}Arg\text{-}CH_2Cl)_2$] were prepared and are potent thrombin inhibitors. These conjugates are model compounds for developing specific thrombus-bound thrombin inhibitors which may have therapeutic application in the treatment of coagulation disorders.

KEY WORDS: Thrombin inhibitors, peptide chloromethyl ketones

INTRODUCTION

Thrombin has a central regulatory role in hemostasis[1,2] and is formed upon cleavage of prothrombin by factor Xa in the blood coagulatlon pathway. Thrombin can cleave fibrinogen to form fibrin clots and can activate factor V, VIII, XIII and protein C which are important in the control of hemostasis and thrombosis. Additionally, thrombin stimulates platelet secretion and aggregation in blood and mediates other nonhemostatic cellular events. Thrombin and other coagulation enzymes are trypsin-like serine proteases, which have the catalytic triad (Asp102, His57 and Ser195) in their active sites and Asp189 in the primary substrate binding site (S_1).[3] Asp189 plays

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an important role in the recognition and binding of substrates and inhibitors to the active site of thrombin and other trypsin-like enzymes.

Since thrombin is a powerful trigger for thrombus formation in blood, it has been targeted along with other coagulation enzymes for the design of new antithrombotic drugs.[4,5,6] Nonpeptidyl inhibitors of thrombin include benzamidine derivatives such as NAPAP[7,8,9] and arginine derivatives such as Argatroban which inhibit thrombin reversibly with K_I values in the submicromolar range.[10] Peptide inhibitors containing arginine aldehydes,[11,12,13] arginine boronic acids,[14,15,16] and arginine trifluoromethyl ketones[17] are transition-state inhibitors of thrombin and the most potent and specific inhibitors contain the thrombin specific D-Phe-Pro-Arg sequence or a related sequence.

Peptide chloromethyl ketones have been widely studied as thrombin inhibitors. Tos-Lys-CH_2Cl (TLCK) and 4-amidinophenylalanine chloromethyl ketone inhibit trypsin and thrombin,[18,19,20] but tripeptide chloromethyl ketones which contain Arg or Lys at the site P_1 and Pro at the site P_2 are more potent thrombin inhibitors.[21,22,23] The P_2 Pro is found at thrombin cleavage sites of prothrombin and factor XIII. D-Phe-Pro-Arg-CH_2Cl (PPACK) is one of the most potent and selective inhibitors of thrombin and is 3-4 orders of magnitude more reactive towards thrombin than other coagulation enzymes.[22,23] Oligopeptide chloromethyl ketones based on the sequence of fibrinopeptide A have also been used to study the interaction of fibrinogen with thrombin.[24] Fluorescent labeled or biotinylated peptide chloromethyl ketones including D-Phe-Pro-Arg-CH_2Cl derivative can discriminate between blood coagulation enzymes and their zymogens.[25] A thioester moiety has been linked to arginine peptide chloromethyl ketones including D-Phe-Pro-Arg-CH_2Cl and the derivatives were used to selectively label thrombin and other coagulation enzymes.[26,27] Fluorescence probes have also been attached to the enzyme through a sulfide linkage for the study of their regulatory interactions.

The inhibitor D-Phe-Pro-Arg-CH_2Cl has been tested in various animal models as an antithrombotic agent. The inhibitor prevents intravascular coagulation caused by thrombin or tissue thromboplastin[28] and appears to prevent thrombin-mediated disseminated intravascular coagulation in dogs.[29] The continuous infusion of D-Phe-Pro-Arg-CH_2Cl abolishes platelet accumulation and occlusion of thrombogenic segments in baboon models of thrombosis.[30] This result indicates that the low molecular weight thrombin inhibitors such as D-Phe-Pro-Arg-CH_2Cl can be useful for treatment of coagulation disorders. At present, there are no ways of targeting low molecular weight thrombin inhibitors to specific thrombogenic sites. We report here the syntheses of conjugates of albumin with bis chloromethyl ketones and their inhibitory potency toward thrombin. The inhibition of thrombin by these conjugates suggests the possibility that other protein-low molecular weight thrombin inhibitor conjugates that may have therapeutic use. We also report the inhibition of thrombin and several coagulation enzymes by other Arg-containing chloromethyl ketones.

MATERIALS AND METHODS

Human thrombin and porcine factor IXa were kindly supplied by Drs. Sriram Krishnaswamy and Pete Lollar, Division of Hematology-Oncology, Emory University School of Medicine. Human factor XIIa, and plasma kallikrein were kindly supplied by Dr. Kazuo Fujikawa, Department of Biochemistry, University of Washington. Human factor VIIa was a generous gift of Dr. George Vlasuk at Merck Sharp and Dohme Research Laboratory. Hepes was purchased from Research Organics Inc., Cleveland, OH. 4,4'-Dithiodipyridine (Aldrithiol-4) was purchased from Aldrich Chemical Co., Milwaukee, WI. Bovine albumin was obtained from Sigma Chemical Co., St Louis, MO, and SP-Sephadex C-25 was purchased from Pharmacia LKB Biotechnology Inc., Piscataway, NJ. All amino acids derivatives were purchased from Bachem Bioscience Inc., Philaldelphia, PA. Z-Arg-SBzl and Z-Trp-Arg-SBzl were synthesized as previously described.[31,32]

Synthesis

Arg(NO_2)-CH_2Cl·HCl, Arg(Tos)-CH_2Cl·HCl, D-Phe-Pro-Arg-CH_2Cl and Dns-Glu-Gly-Arg-CH_2Cl were synthesized by a previously described method.[23] Boc-Phe-Phe-OH, Boc-Phe-D-Phe-Pro-OH, and Boc-Gly-D-Phe-Pro-OH were prepared using the standard DCC/HOBt coupling method. The syntheses of other peptide chloromethyl ketones are described below.

N-Benzyloxycarbonyl-arginyl chloromethyl ketone hydrochloride [Z-Arg-CH_2Cl·HCl, **1***].* Arg(NO_2)-CH_2Cl·HCl (0.23 g, 0.8 mmol) was treated with anhydrous HF (ca. 10 mL) in the presence of anisole (1.0 mL) for 30 min at 0°C. After removal of HF, H_2O was added to the residue and the solution was washed 3 times with ether. The aqueous layer was applied to a column containing 10 mL of SP-Sephadex (C-25, H^+) and was washed with H_2O (100 mL). The product, Arg-CH_2Cl·2HCl was eluted from the Sephadex with 0.5 N HCl (100 mL) and the eluted solution was concentrated *in vacuo*. The residue was dried completely *in vacuo* and used for the next reaction without further purification.

To a DMF solution (4 mL) of Arg-CH_2Cl·2HCl (0.8 mmol) was added triethylamine (0.22 mL, 1.6 mmol) and benzyl chloroformate (0.13 mL, 0.8 mmol) at −15°C. After stirring for 1 h, the reaction mixture was concentrated *in vacuo*. Ethyl acetate was added to the residue and the organic layer was washed with saturated NaCl solution twice. The organic portion was dried over $MgSO_4$ and the solvent was removed by evaporation. The crude product was purified by silica gel chromatography which was eluted with $CHCl_3$:CH_3OH = 10:1 and triturated with ether to give the final product (0.11 g, 36% yield) as a hygroscopic yellowish powder; R_f = 0.56 ($CHCl_3$:CH_3OH:CH_3CO_2H = 80:10:5). Anal. (exact mass, HRMS) calcd. for $C_{15}H_{22}N_4O_3Cl_1$ m/e 341.1380, found 341.1616.

Phenylalanylphenylalanylarginyl chloromethyl ketone hydrochloride [Phe-Phe-Arg-CH_2Cl·2HCl, **2***].* Boc-Phe-Phe-Arg(Tos)-CH_2Cl was prepared by the mixed anhydride coupling method from Boc-Phe-Phe-OH and Arg(Tos)-CH_2Cl·HCl. To a THF

solution (20 mL) of Boc-Phe-Phe-OH (0.41 g, 1.0 mmol) was added successively N-methylmorpholine (0.11 mL, 1.0 mmol) and isobutyl chloroformate (0.13 mL, 1.0 mmol) at −15°C. After stirring for 2 min and addition of cold THF solution (2 mL) of triethylamine (0.15 mL, 1.1 mmol), the mixture was added to a DMF solution (1 mL) of Arg(Tos)-CH_2Cl·HCl (0.44 g, 1.1 mmol) at −15°C. After stirring at −15°C for 1 h and at 0–5°C for 12 h, the reaction mixture was concentrated *in vacuo*. Ethyl acetate was added to the residue and the solution was washed successively with 1 N HCl, 5% $NaHCO_3$ and a saturated NaCl solution. The organic layer was dried over Na_2SO_4 and the solvent was removed by evaporation. The crude product was purified by silica gel chromatography which was eluded with $CHCl_3$:CH_3OH = 50:1 and then reprecipitated with $CHCl_3$-ether to give the final product (0.25 g, 33% yield) as a white powder. Anal. (exact mass, HRMS) calcd. for $C_{37}H_{47}N_6O_7S_1Cl$ m/e 755.2993, found, 755.3019. Boc-Phe-Phe-Arg(Tos)-CH_2Cl (0.22 g, 0.29 mmol) was treated with HF by the same procedure described for Arg-CH_2Cl·2HCl. The final product was obtained as a yellowish powder (yield 90%). Anal. (exact mass, HRMS) calcd. for $C_{25}H_{34}N_6O_3Cl_1$ m/e 501.2380, found 501.2380.

Phenylalanyl-D-phenylalanylprolylarginyl chloromethyl ketone hydrochloride [Phe-D-Phe-Pro-Arg-CH_2Cl·2HCl, **4***].* Boc-Phe-D-Phe-Pro-Arg(NO_2)-CH_2Cl was prepared by the mixed anhydride method from Boc-Phe-D-Phe-Pro-OH and Arg(NO_2)-CH_2 Cl·HCl. The crude product purified by silica gel chromatography and was eluted with $CHCl_3$:CH_3OH = 30:1 to give the final product (0.57 g, 70% yield) as a white powder. The deblocked derivative, Phe-D-Phe-Pro-Arg-CH_2Cl was obtained by treatment with HF as a pale yellow powder (0.37 g, 90%). Anal. (exact mass, HRMS) calcd. for $C_{30}H_{41}N_7O_4Cl_1$ m/e 598.2908, found 598.2903.

Glycyl-D-phenylalanylprolylarginyl chloromethyl ketone hydrochloride [Gly-D-Phe-Pro-Arg-CH_2Cl·2HCl, **5***].* Boc-Gly-D-Phe-Pro-Arg(NO_2)-CH_2Cl was synthesized from Boc-Gly-D-Phe-Pro-OH and Arg(NO_2)-CH_2Cl·HCl by the mixed anhydride coupling method. The crude product was purified by silica gel column chromatography ($CHCl_3$:CH_3OH = 25:1) to give the final product as a white powder (0.60 g, 83% yield). Boc-Phe-D-Phe-Pro-Arg(NO_2)-CH_2Cl (0.40 g, 0.61 mmol) was treated with HF to give H-Gly-D-Phe-Pro-Arg-CH_2Cl·2Hl as a yellowish powder (0.22 g, 62%). Anal. (Exact mass, HRMS) calcd. for $C_{23}H_{35}N_7O_4Cl_1$ m/e 508.2439, found 508.2957.

D-Phenylalanylprolyl-(ω-tosyl)arginyl chloromethyl ketone hydrochloride [D-Phe-Pro-Arg(Tos)-CH_2Cl·HCl]. Boc-D-Phe-Pro-Arg(Tos)-CH_2Cl was prepared from Boc-D-Phe-Pro-OH and Arg(Tos)-CH_2Cl·HCl by the mixed anhydride coupling method. The crude product was purified by silica gel column chromatography ($CHCl_3$:CH_3OH = 35:1) and trituration with ether to give the final product as a white powder (0.3 g, 53% yield). An ethyl acetate solution (15 mL) saturated with HCl was added to Boc-D-Phe-Pro-Arg(Tos)-CH_2Cl (0.25 g, 0.35 mmol) at 0°C and the solution was stirred at r.t. for 1 h. The solvent was removed *in vacuo* and the residue was triturated with ethyl acetate to give the final product as a white powder (0.22 g, 98%).

Oxalyl-bis(D-phenylalanylprolylarginyl chloromethyl ketone) dihydrochloride [(CO-D-Phe-Pro-Arg-CH_2Cl·HCl)$_2$, oxalyl-CMK, **6***].* Triethylamine (0.1 mL, 0.72 mmol) and

oxalyl chloride (16 μL, 0.18 mmol) were added to a THF (3 mL) solution of D-Phe-Pro-Arg(Tos)-CH_2Cl·HCl (0.22 g, 0.34 mm) at −10°C. After stirring at r.t. for 3 h, the reaction mixture was filtered and the filtrate was concentrated *in vacuo* and solidified with ether. The crude product was purified by silica gel chromatography using $CHCl_3$:CH_3OH = 30:1 as an eluant and triturated with ether to give the final product (CO-D-Phe-Pro-Arg(Tos)-$CH_2Cl)_2$ as a white powder (75 mg, 17% yield); TLC ($CHCl_3$:CH_3OH:AcOH = 95:5:3) R_f = 0.51; MS (FAB) m/e 1263 $(M + 1)^+$. The intermediate (CO-D-Phe-Pro-Arg(Tos)-$CH_2Cl)_2$ (70 mg, 55 μmol) was treated with HF to give the final compound as a slightly yellowish powder (52 mg, 91%); TLC ($CHCl_3$:CH_3OH:AcOH = 5:2:1) R_f = 0.12; MS (FAB) m/e 956 $(M - 2HCl)^+$. Anal. (exact mass, HRMS) calcd. for $C_{44}H_{62}N_{12}O_8Cl_2$ m/e 956.4193, found 956.4190.

Glutaryl-bis(D-phenylalanylprolylarginyl chloromethyl ketone) dihydrochloride [$(CH_2)_3$-(CO-D-Phe-Pro-Arg-[$(CH_2Cl$·$HCl)_2$, glutaryl-CMK, ***7****].* The intermediate $(CH_2)_3$-(CO-D-Phe-Pro-Arg(Tos)-$CH_2Cl)_2$ was prepared by adding triethylamine (0.12 mL, 0.85 mmol) and glutaryl dichloride (28 μL, 0.21 mmol) to a THF (3 mL) solution of D-Phe-Pro-Arg(Tos)-CH_2Cl·HCl (0.22 g, 0.34 mmol) at -10°C. After stirring at r.t. for 3 h, the reaction mixture was filtered and the filtrate was concentrated *in vacuo* and solidified with ether. The crude powder was purified by silica gel chromatography using $CHCl_3$:CH_3OH = 20:1 as an eluant and triturated with ether to give the final product (35 mg, 8% yield) as a white powder; TLC ($CHCl_3$:CH_3OH:AcOH = 80:10:5) R_f = 0.69; MS (FAB) m/e 1305 $(M + H)^+$. Anal. (Exact mass, HRMS) calcd. for $C_{61}H_{79}N_{12}O_{12}S_2Cl_2$ m/e 1305.4759, found 1305.5697. The intermediate $(CH_2)_3$-(CO-D-Phe-Pro-Arg(Tos)-$CH_2Cl)_2$ (35 mg, 28 μmol) was then treated with HF (ca. 5 mL) to give the final compound as a slightly yellowish powder (27 mg, 94%); TLC ($CHCl_3$:CH_3OH:AcOH = 5:2:1) R_f = 0.12. MS (FAB) m/e 997 $(M - 2HCl + H)^+$. Anal. (exact mass, HRMS) calcd. for $C_{47}H_{67}N_{12}O_8Cl_2$ m/e 997.4582, found 997.5563.

Conjugates of Albumin with (CO-D-Phe-Pro-Arg-$CH_2Cl)_2$ and $(CH_2)_3$-(CO-D-Phe-Pro-Arg-$CH_2Cl)_2$ (Albumin-S-CH_2-Arg<-Proc<-D-Phe<-CO-CO-D-Phe-Pro-Arg-CH_2 Cl ***[8]****, Albumin-S-CH_2-Arg<-Pro<-D-Phe<-CO-$(CH_2)_3$-CO-D-Phe-Pro-Arg-CH_2Cl* ***[9]****).* Bovine albumin (100 mg in 0.1 M $NaHCO_3$, pH 8.1 buffer, 146 μM by absorbance at 280 nm) was titrated with 4,4'-dithiodipyridine and only 49 μM of thiol group was found. This albumin solution was incubated with **6** or **7** (0.91 mM) in 0.1 M $NaHCO_3$, pH 8.1 buffer at r.t. for 10–120 min. Only 40% of the thiol groups (19 μM) in albumin reacted with the oxalyl-CMK **6** and 45% of the thiol groups (22 μM) reacted with the glutaryl-CMK **7**. Excess inhibitor was removed from the diluted albumin solutions by centrifugation three times using Amicon centricon-10 microconcentrators and gel filtration once.

Enzyme Inactivation – Incubation Method

An aliquot of inhibitor (25–50 μL) in Me_2SO was added to 0.275–0.55 mL of a buffered enzyme solution (0.03–4.3 μM) to initiate the inactivation reaction. Aliquots (50–500 μL) were withdrawn at various time intervals and the residual enzymatic activity was measured as described below. Enzymatic activities of human

TABLE 1
Inhibition Constants of Coagulation Enzymes by Peptide Chloromethyl Ketones.[a]

Compounds	$k_{obs}/[I]$ ($M^{-1}s^{-1}$)		
	human thrombin[b]	porcine factor IXa[c]	human plasma kallikrein[d]
Z-Arg-CH_2Cl (**1**)	160	NI[e]	440
Phe-Phe-Arg-CH_2Cl (**2**)	25	1.4	3110
D-Phe-Pro-Arg-CH_2Cl (**3**)	10,400,000[f] (11,500,000)[g]	20	5090 (7900)[g]
Phe-D-Phe-Pro-Arg-CH_2Cl (**4**)	275,000	2	640
Gly-D-Phe-Pro-Arg-CH_2Cl (**5**)	100,000	3	950
(CO-D-Phe-Pro-Arg-$CH_2Cl)_2$ (**6**)	665,000		
$(CH_2)_3$-(CO-D-Phe-Pro-Arg-$CH_2Cl)_2$ (**7**)	179,000		
albumin-oxalyl CMK conjugate (**8**)	79,000		
albumin-glutaryl CMK conjugate (**9**)	29,000		

[a] Inhibition constants were measured in 0.1 M Hepes, 0.01 M $CaCl_2$, pH 7.5 buffer containing 8% Me_2SO and at 23°C. [b] Inhibitor concentrations were: **1**, 45 μM; **2**, 430 μM; **3**, 0.032 μM; **4**, 0.42 μM; **5**, 0.21 μM; **6**, 0.21 μM; **7**, 0.21 μM; **8**, 1.63 μM; **9**, 0.74 μM. [c] Inhibitor concentrations were: **1**, 450 μM; **2**, 430 μM; **3**, 400 μM; **4**, 423 μM; **5**, 427 μM. [d] Inhibitor concentrations were: **1**, 45 μM; **2**, 8.7 μM ; **3**, 4.2 μM; **4**, 4.2 μM; **5**, 8.5 μM. [e] No inhibition after 10 min incubation. [f] The second order inhibition constant (k_{2nd}) was obtained at equimolar concentration of enzyme and inhibitor. [g] Data was obtained from reference 23. The inhibition reaction was conducted at pH 7.0 and 25°C, bovine thrombin was used.

thrombin, human factor Xa, human factor XIIa and porcine factor IXa were measured in 0.1 M Hepes, 0.01 M $CaCl_2$, pH 7.5 buffer. Enzymatic activity of human factor VIIa was measured in 0.05 M Hepes, 0.15 M NaCl, 0.005 M $CaCl_2$, pH 7.5 buffer. The Me_2SO concentration in the reaction mixtures was 8–12% (v/v). The inhibitor concentrations are shown in Table 1. Porcine factor IXa was assayed with Z-Trp-Arg-SBzl (0.157 mM), and other coagulation enzymes were assayed with Z-Arg-SBzl (0.07–0.133 mM). All peptide thioester hydrolysis rates were measured with assay mixtures containing 4,4'-dithiodipyridine (ϵ_{324} = 19800 $M^{-1}cm^{-1}$).[33] Pseudo first-order inactivation rate constants (k_{obs}) were obtained from plots of ln v_t/v_o *vs* time, and the correlation coefficients were greater than 0.98. The inactivation reactions were followed for 2–3 half-lives and each kinetic experiment was run at least twice.

Stabilities of Oxalyl-CMK and Albumin-Oxalyl CMK Conjugate

The decrease of inhibitory activity of oxalyl-CMK (**6**) toward thrombin was used to measure its stability in 0.1 M $NaHCO_3$; pH 8.1 buffer. Aliquots (10 μL) of inhibitor

(0.1 mM) were added to 0.26 mL of thrombin-containing (0.03 μM) buffer solution to inhibit the enzyme completely, then aliquots (25 μL) were withdrawn at various time intervals to measure the thrombin activities using Z-Arg-SBzl (0.1 mM) as the substrate until full enzyme activity was obtained. Pseudo first-order inactivation rate constants (k_{obs}) were obtained from plots of ln $(v_f\text{-}v_t)/v_f$ *vs* time, where v_t and v_f were initial rates at time *t* and at time when full activity obtained. The correlation coefficients were greater than 0.98.

The decrease of inhibitory activity of albumin-oxalyl CMK conjugate (**8**) toward trypsin was used to measure its stability in 0.1 M Hepes, 0.01 M $CaCl_2$, pH 7.5 buffer. Aliquots (10 μL) of inhibitor (20 μM) were added to 0.26 mL of trypsin-containing (0.07 μM) buffer solution to inhibit the enzyme, then aliquots (25 μL) were withdrawn at various time intervals to measure the trypsin activities until full enzyme activity was obtained. Pseudo first-order inactivation rate constants (k_{obs}) were obtained from plots of ln $(v_f\text{-}v_t)/v_f$ *vs* time, where v_t and v_f were initial rates at time *t* and at time when full activity obtained. The correlation coefficients were greater than 0.98.

RESULTS AND DISCUSSION

Thrombus formation often occurs in patients with myocardial infarction and other coagulation disorders. Thrombin and factor Xa form rapidly at highly thrombogenic sites and activate platelets and cleave fibrinogen in the developing thrombus. One way to target low molecular weight inhibitors to thrombus-bound thrombin would involve the attachment of inhibitors to proteins or other macromolecules which specifically bind to thrombi. For example, it should be possible to attach low molecular weight inhibitors to antibodies specific for fibrin, platelets or other macromolecules associated with thrombi. Such conjugates potentially would inhibit thrombus-bound thrombin but not affect thrombin at other sites. This would allow control of thrombogenesis but have no effect on hemostasis.

One method to prepare protein-thrombin inhibitor conjugates is shown in Figure 1. The inhibitors are bis chloromethyl ketones where two molecules of D-Phe-Pro-Arg-CH_2Cl are linked with a bifunctional spacer such as an oxalyl or glutaryl group. Proteins such as albumin and an anti-fibrin antibody fragment which contain one thiol group can easily react with the chloromethyl ketone functional group of the inhibitor to form a covalent linkage. The remaining chloromethyl ketone moiety at the other end of the protein-inhibitor conjugate can then inactivate thrombin. One previous example is the conjugate of albumin microsphere and Suc-Ala-Ala-Pro-Val-CH_2Cl which inhibits human leukocyte elastase potently and was thought to have therapeutic application in the treatment of emphysema.[34]

Arginine-Containing Peptide Chloromethyl Ketones

Two bis chloromethyl ketones, oxalyl-CMK (**6**) and glutaryl-CMK (**7**) were synthesized by coupling D-Phe-Pro-Arg(Tos)-CH_2Cl with oxalyl chloride or glutaryl chloride in the presence of triethylamine to give the intermediate (CO-D-Phe-Pro-Arg(Tos)$CH_2Cl)_2$ or $(CH_2)_3$-(CO-D-Phe-Pro-Arg(Tos)$CH_2Cl)_2$ followed by

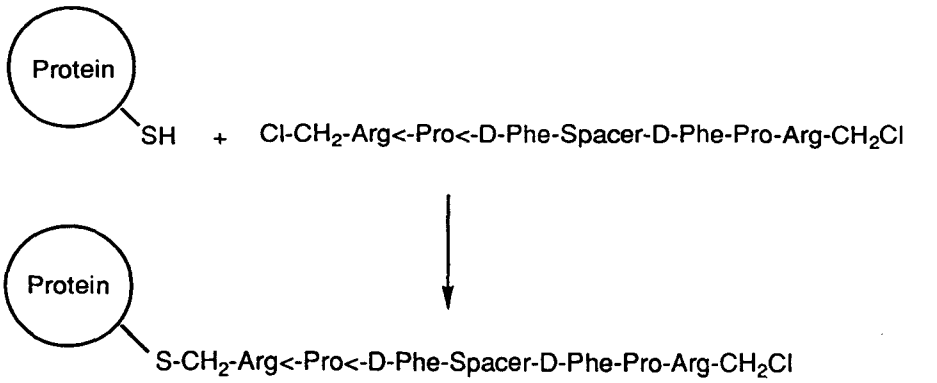

FIGURE 1 Reaction of a thiol-containing protein with a bis chloromethyl ketone to form a protein-peptide chloromethyl ketone conjugate. Two molecules of D-Phe-Pro-Arg-CH_2Cl are linked with a bifunctional spacer (Spacer) to give the bis chloromethyl ketone. The symbol < – indicates a reversed amino acid residue (-CO-CHR-NH-). Spacer represents a group such as an oxalyl (-CO-CO-) or glutaryl (-CO-CH_2-CH_2-CH_2-CO-) group.

treatment with HF. Other Arg-containing peptide chloromethyl ketones were obtained by treatment of Arg(NO_2)- or Arg(Tos)-containing precursors with HF. The final products were usually purified by SP-Sephadex column chromatography.

Inhibition of thrombin, factor IXa and plasma kallikrein by Arg-containing chloromethyl ketones is shown in Table 1. The most potent thrombin inhibitor D-Phe-Pro-Arg-CH_2Cl (**3**) has been reported to inhibit factor Xa and plasma kallikrein less effectively than thrombin by 3 orders of magnitude.[23] This compound inhibits factor IXa weakly with the inhibition rate ca. 520,000 fold less than that of thrombin (Table 1). Human factor VIIa and XIIa are also inhibited by D-Phe-Pro-Arg-CH_2Cl with $k_{obs}/[I]$ values of 110 (at 40 μM) and 665 $M^{-1}s^{-1}$ (at 42 μM), respectively. This result indicates that D-Phe-Pro-Arg-CH_2Cl is quite specific toward thrombin when compared with other coagulation enzymes.

Extension by one amino acid (Gly or Phe) or a peptide moiety ($ClCH_2$-Arg<-Pro<-D-Phe<-CO-CO-, $ClCH_2$-Arg<-Pro<-D-Phe<-CO-$(CH_2)_3$-CO-, where <- indicates a reversed amino acid residue, -CO-CHR-NH-) on the N-terminus of D-Phe-Pro-Arg-CH_2Cl (**4–7**) reduced the thrombin inhibition constants by 1–2 orders of magnitude, which indicates the free amino group at the N-terminus is important for the inhibition. This result is consistent with the x-ray structure of the α-thrombin and D-Phe-Pro-Arg-CH_2Cl complex where the N-terminal amino group of the inhibitor forms hydrogen bonding with the carbonyl of Gly216.[35] The tetrapeptide **4** with Phe at the P_4 site is a better thrombin inhibitor than the Gly derivative **5**. The bis chloromethyl and compounds oxalyl-CMK **6** inhibits thrombin more potently than the glutaryl-CMK **7** by 4 fold probably because compound **7** contains three extra methylene groups between two D-Phe-Pro-Arg units and is more flexible than the oxalyl derivative **6**.

Two chloromethyl ketones Z-Arg-CH_2Cl (**1**) and Phe-Phe-Arg-CH_2Cl (**2**) inhibited

thrombin very poorly when compared to D-Phe-Pro-Arg-CH_2Cl (**3**), which can be readily explained from the crystal structure of human α-thrombin and D-Phe-Pro-Arg-CH_2Cl complex.[35] In the enzyme-inhibitor structure, the P_2 proline of the inhibitor is encapsulated in a very hydrophobic cage formed by the side chains of Trp215, Leu99, His57, Tyr60A and Trp60D, and the P_2 benzyl group of Z-Arg-CH_2Cl and Phe-Phe-Arg-CH_2Cl is too big to fit into this pocket. The P_3 D-Phe occupied a second hydrophobic pocket surrounded by Ile174, Trp215, segment 97-99 and Tyr60A, and the P_3 benzyl group of Phe-Phe-Arg-CH_2Cl cannot fit into this pocket due to the incorrect configuration of L-Phe at this site in the inhibitor.

All the peptide chloromethyl ketones inhibit factor IXa poorly with $k_{obs}/[I]$ values less than 20 $M^{-1}s^{-1}$, however they inhibit plasma kallikrein moderately with $k_{obs}/[I]$ values of 10^2–10^3 $M^{-1}s^{-1}$. Dns-Glu-Gly-Arg-CH_2Cl, a potent factor Xa inhibitor, inhibits human factor VIIa and XIIa poorly with $k_{obs}/[I]$ values of 16 (at 400 μM) and 130 $M^{-1}s^{-1}$ (42 μM), respectively.

Bis Chloromethyl Ketone-Albumin Conjugates

Conjugates of albumin wlth oxalyl-CMK and glutaryl-CMK were prepared by incubating albumin with chloromethyl ketones in pH 8.1 buffer for 10–120 min. Only a fraction (40–45%) of the thiol groups reacted with the bis chloromethyl ketones even when the reaction time was increased from 10 to 120 min and the reaction mixture contained excess inhibitors ([I]/[albumin-SH] = 19/1]. The bis compound oxalyl-CMK (**6**) was hydrolyzed in a pH 8.1 buffer with half-life of 80 min while the albumin-oxalyl CMK conjugate (**8**) had a half-life of 4,300 min in a pH 7.5 buffer, which indicates the chloromethyl ketone is less stable in the basic buffer. The inhibitory potency of these conjugates toward thrombin was compared with other Arg-containing chloromethyl ketones (Table 1). The conjugates albumin-oxalyl CMK (**8**) and albumin-glutaryl CMK (**9**) are potent thrombin inhibitors with $k_{obs}/[I]$ values in the order of 10^4 $M^{-1}s^{-1}$, although they inhibited thrombin less potently than D-Phe-Pro-Arg-CH_2Cl (**3**) by 130–360 fold. These two conjugates also inhibited thrombin less effectively than the bis chloromethyl ketones (**6** and **7**) by 6–8 fold. However, they are much better thrombin inhibitors than Z-Arg-CH_2Cl (**1**) and Phe-Phe-Arg-CH_2Cl (**2**). The albumin-oxalyl CMK conjugate **8** inhibits thrombin more potently than the albumin-glutaryl CMK **9** since the former is less flexible than the latter which contains three extra methylene spacer groups between two D-Phe-Pro-Arg units.

CONCLUSION

Arg-containing peptide chloromethyl ketones including D-Phe-Pro-Arg-CH_2Cl derivatives inhibit thrombin and several blood coagulation enzymes. D-Phe-Pro-Arg-CH_2Cl is still the best thrombin inhibitor in the series. The tetrapeptide and bis derivatives of D-Phe-Pro-Arg-CH_2Cl are potent thrombin inhibitors but they are less effective than the parent compound, which indicates that the free amino group at the N-terminus is important for the inhibition. Z-Arg-CH_2Cl and Phe-Phe-Arg-CH_2Cl which have a large hydrophobic group at the P_2 site inhibit thrombin poorly when

compared to D-Phe-Pro-Arg-CH_2Cl. All the peptide chloromethyl ketones inhibit plasma kallikrein moderately but inhibit factor IXa poorly. Conjugates of albumin with bis chloromethyl ketones were prepared and were potent thrombin inhibitors. The success of this conjugate model system suggests the possibility of preparing other protein-thrombin inhibitor conjugates which can specifically bind to thrombi and simultaneously inhibit thrombin.

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3. The nomenclature of Schecher and Berger (1967) *Biochem. Biophys. Res. Commun.*, **27**, 157–152, is used to designate the individual amino acid residues (P_2, P_1, P_1', P_2', etc.) of a peptide substrate and the corresponding subsites (S_2, S_1, S_1', S_2', etc.) of the enzyme. The P_1-P_1' peptide bond is the scissile bond.
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7. Abbreviations: Argatroban, (2R,4R)-4-methyl-1-[N^{α}-[(3-methyl-1,2,3,4,-tetrahydro-8-quinolinyl)-sulfonyl]-L-arginyl]-2-piperidinecarboxylic acid; Boc, *t*-butyloxycarbonyl; CMK, chloromethyl ketone; DCC, 1,3-dicyclohexylcarbodiimide; DMF, N,N-dimethylformamide; Dns, dansyl (5-dimethylamino-naphthalenesulfonyl); Hepes, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; HOBt, 1-hydroxy-benzotriazole; NAPAP, N^{α}-(2-naphthylsulfonylglycyl)-DL-4-amidinophenylalanylpiperidine; SBzl, thiobenzyl ester; Suc, succinyl; THF, tetrahydrofuran; Tos, *p*-toluene sulfonyl; Z, benzyloxycarbonyl.
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