Biochimica et Biophysica Acta, 484 (1977) 417—422

BBA 68238

SYNTHETIC INHIBITORS OF TRYPSIN, PLASMIN, KALLIKREIN, THROMBIN, $C_1\bar{r}$, AND C_1 ESTERASE

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(Received September 21st, 1976)
(Revised manuscript received May 10th, 1977)

Summary

p-Carbethoxyphenyl ϵ-guanidinocaproate and *p*-(*p'*-guanidinobenzoyloxy)-phenyl derivatives were prepared, and their inhibitory effects on trypsin, plasmin, plasma kallikrein, thrombin, $C_1\bar{r}$ and C_1 esterase were examined. Among the various inhibitors tested, *p*-nitrophenyl *p'*-guanidinobenzoate, *N,N*-dimethylamino *p*-(*p'*-guanidinobenzoyloxy)-benzoyl glycolate and *N,N*-dimethylamino *p*-(*p'*-guanidinobenzoyloxy)-benzilcarbonyloxy glycolate were the most effective inhibitors of trypsin, plasmin, plasma kallikrein and thrombin, and they strongly inhibited the esterolytic activities of $C_1\bar{r}$ and C_1 esterase.

Introduction

Previously [1,2], we reported the strong, reversible inhibitory effects of various ω-guanidino acid esters on trypsin, plasmin, plasma kallikrein and thrombin. Among the various esters examined, aromatic esters, such as *p*-carbethoxyphenyl ϵ-guanidinocaproate monophosphate, were more inhibitory than aliphatic esters. Moreover, aromatic esters of ϵ-guanidinocaproic acid and *trans*-4-aminomethylcyclohexane-carboxylicacid (AMCHA) caused strong, competitive inhibition of C_1 esterase and the conversion of C_1 to C_1 esterase and the strongest inhibitor was the phenyl ester of ϵ-guanidinocaproic acid [3].

Mares-Guia and Shaw [4] reported the irreversible inhibitory effect of ethyl

Abbreviations: TosArgOMe, N^{α}-tosyl-L-arginine methyl ester; *N*-AcTyrOEt, N^{α}-acetyl-L-tyrosine ethyl ester; *N*-AcArgOMe, N^{α}-acetyl-L-arginine methyl ester; AMCHA, *trans*-4-aminomethylcyclohexane-carboxylic acid; ϵ-GCA-CEP, *p*-carbethoxyphenyl ϵ-guanidinocaproate; *p'*-GB-NP, *p*-nitrophenyl *p'*-guanidinobenzoate; *p'*-GB-DBoG, *N,N*-dimethylamino *p*-(*p'*-guanidinobenzoyloxy)-benzoyl glycolate; *p'*-GB-DBiG, *N,N*-dimethylamino *p*-(*p'*-guanidinobenzoyloxy)-benzilcarbonyloxy glycolate.

p-guanidinobenzoate on trypsin, and Chase and Shaw [5] reported that of *p*-nitrophenyl-*p'*-guanidinobenzoate. Bing [6] reported that a highly purified subunit of activated human complement, component C_1 ($C_1\bar{s}$), hydrolyzed *p*-nitrophenyl N^{α}-carbobenzoxy-L-tyrosinate, and that the reaction was inhibited competitively by guanidine, amidine and a variety of aromatic compounds of low molecular weight.

This paper reports that *p*-(*p'*-guanidinobenzoyloxy)-phenyl derivatives are stronger inhibitors of trypsin, plasmin, plasma kallikrein and thrombin than *p*-carbethoxyphenyl ϵ-guanidinocaproate monophosphate, and that they inhibit C_1 esterase as much as the phenyl ester of ϵ-guanidinocaproic acid. Studies on the inhibitory effects of these compounds on $C_1\bar{r}$, a subcomponent of the first component of human complement are also described.

Materials and Methods

Enzymes

Human plasmin [7], plasma kallikrein [7], C_1 esterase [8] and $C_1\bar{r}$ [9] were prepared as described previously. Bovine trypsin (type I) was purchased from Sigma Chemical Co., and dissolved in 0.1 M sodium phosphate buffer, pH 7.4. Human thrombin was purchased from the Green Cross Corporation, Osaka, Japan and dissolved in 0.1 M sodium phosphate buffer, pH 7.4.

Substrates and inhibitors

N^{α}-Tosyl-L-arginine methyl ester (TosArgOMe), N^{α}-acetyl-L-tyrosine ethyl ester (*N*-AcTyrOEt), and N^{α}-acetyl-L-arginine methyl ester (*N*-AcArgOMe) were purchased from the Foundation for Promotion of Protein Research, Institute for Protein Research, Osaka University, Osaka, Japan. Casein was purchased from E. Merck Japan Ltd, and bovine fibrinogen (fraction I) from Sigma Chemical Co. *p*-Carbethoxyphenyl ϵ-guanidinocaproate (ϵ-GCA-CEP), *p*-nitrophenyl *p'*-guanidinobenzoate (*p'*-GB-NP), *N*,*N*-dimethylamino *p*-(*p'*-guanidinobenzoyloxy)-benzoyl glycolate (*p'*-GB-DBoG) and *N*,*N*-dimethylamino *p*-(*p'*-guani-

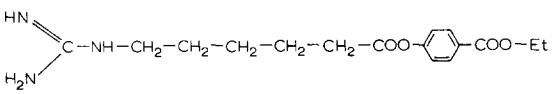
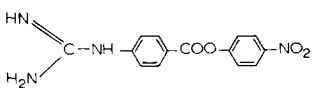
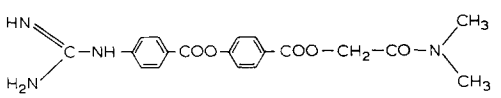
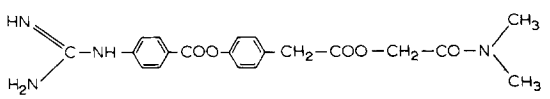
Compound	Structure
ϵ-GCA-CEP	
p'-GB-NP	
p'-GB-DBoG	
p'-GB-DBiG	

Fig. 1. Structural formulae of ϵ-GCA-CEP, *p'*-GB-NP, *p'*-GB-DBoG, and *p'*-GB-DBiG.

dinobenzoyloxy)-benzilcarbonyloxy glycolate (*p'*-GB-DBiG) were prepared in the Research Laboratories of Ono Pharmaceutical Company, Osaka, Japan. The structural formulas of these compounds are shown in Fig. 1.

Experiments on inhibition of various enzymes

The rates of hydrolysis of TosArgOMe by trypsin, plasmin, plasma kallikrein and thrombin were determined as described previously [10], at a substrate concentration of 10 mM; The rates of hydrolysis of *N*-AcArgOMe by $C_1\bar{r}$ and *N*-AcTyrOEt by C_1 esterase were determined as described previously [9], at a substrate concentration of 10 mM. The caseinolysis of trypsin and fibrinogenolysis of plasmin were determined as described previously [7]. The final concentrations of both casein and fibrinogen were 2%. To measure inhibitor, mixtures of enzyme solution and inhibitor were preincubated at 37°C for 5 min and then residual enzyme activities were determined, as described above.

The K_m values were determined from the Lineweaver-Burk [11] plot of the results. The K_i values were determined as described by Dixon [12].

Results and Discussion

The effects of ε-GCA-CEP and *p*-(*p'*-guanidinobenzoyloxy)-phenyl derivatives on the esterolysis of various enzymes were examined, and the concentrations required for 50% inhibition are shown in Table I. The concentrations of *p'*-GB-DBiG required for 50% inhibition of the caseinolysis of trypsin and fibrinogenolysis of plasmin were $3.0 \cdot 10^{-8}$ M and $2.5 \cdot 10^{-7}$ M, respectively. This indicated that the intensity of the inhibitor activity on esterolysis and proteolysis was almost the same.

Previously [2], we examined the inhibitory effects of various esters of ω-guanidino acids on trypsin, plasmin, plasma kallikrein and thrombin and found that among the various inhibitors tested, phenyl and *p*-carbethoxyphenyl ε-guanidinocaproate were the most inhibitory to these four enzymes.

On the other hand, *p*-(*p'*-guanidinobenzoyloxy)-phenyl derivatives inhibited these four enzymes more than ε-GCA-CEP, as shown in Table I. *p'*-GB-DBoG strongly inhibited trypsin, plasmin and thrombin: the concentrations causing 50% inhibition of TosArgOMe hydrolysis by trypsin, plasmin and thrombin were $1.95 \cdot 10^{-9}$, $3.6 \cdot 10^{-8}$, $1.2 \cdot 10^{-6}$ M, respectively. The concentration of

TABLE I

CONCENTRATIONS OF INHIBITORS FOR 50% INHIBITION OF ESTEROLYSIS

Incubations were carried out in 0.1 M sodium phosphate buffer, pH 7.4, at 37°C. TosArgOMe (10 mM) was used as substrate for trypsin, plasmin, kallikrein, and thrombin, *N*-AcArgOMe (10 mM) for $C_1\bar{r}$; and *N*-AcTyrOEt (10 mM) for C_1 esterase.

Inhibitor	Inhibitor concentration for 50% inhibition (M)					
	Trypsin	Plasmin	Plasma kallikrein	Thrombin	$C_1\bar{r}$	C_1 esterase
ε-GCA-CEP	$9.4 \cdot 10^{-6}$	$3.0 \cdot 10^{-5}$	$4.1 \cdot 10^{-5}$	$1.1 \cdot 10^{-4}$		$5.2 \cdot 10^{-4}$
p'-GB-NP	$2.7 \cdot 10^{-8}$	$4.3 \cdot 10^{-8}$	$3.8 \cdot 10^{-7}$	$2.4 \cdot 10^{-6}$		
p'-GB-DBoG	$1.95 \cdot 10^{-9}$	$3.6 \cdot 10^{-8}$		$1.2 \cdot 10^{-6}$		$3.0 \cdot 10^{-5}$
p'-GB-DBiG	$3.2 \cdot 10^{-8}$	$1.9 \cdot 10^{-7}$	$3.2 \cdot 10^{-7}$	$5.0 \cdot 10^{-5}$	$4.4 \cdot 10^{-6}$	$3.4 \cdot 10^{-5}$

TABLE II

K_i VALUES FOR TRYPSIN, PLASMIN, $C_1\bar{r}$ AND C_1 ESTERASE

K_i values were estimated in 0.1 M sodium phosphate buffer, pH 7.4, at 37°C. TosArgOMe was used as substrate for trypsin and plasmin, *N*-AcArgOMe for $C_1\bar{r}$, and *N*-AcTyrOEt for C_1 esterase.

Inhibitor	K_i (M)			
	Trypsin	Plasmin	$C_1\bar{r}$	C_1 esterase
ϵ-GCA-CEP	$2.6 \cdot 10^{-6}$			
p'-GB-DBoG	$8.1 \cdot 10^{-7}$			
p'-GB-DBiG	$3.3 \cdot 10^{-8}$	$3.8 \cdot 10^{-8}$	$5.0 \cdot 10^{-8}$	$1.2 \cdot 10^{-4}$

p'-GB-DBiG for 50% inhibition of TosArgOMe hydrolysis by plasma kallikrein was $3.2 \cdot 10^{-7}$ M. On the other hand, the concentrations of ϵ-GCA-CEP for 50% inhibition of TosArgOMe hydrolysis by trypsin, plasmin, plasma kallikrein and thrombin were $9.4 \cdot 10^{-6}$, $3.0 \cdot 10^{-5}$, $4.1 \cdot 10^{-5}$ and $1.1 \cdot 10^{-4}$ M, respectively, as shown in Table I.

Previously, we reported [3] that aromatic esters of ϵ-guanidinocaproic acid and *trans*-4-aminomethylcyclohexane carboxylic acid (AMCHA) caused strong, competitive inhibition of C_1 esterase and the conversion of C_1 to C_1 esterase. The phenyl ester of ϵ-guanidinocaproic acid was the strongest inhibitor and the concentration causing 50% inhibition of *N*-AcTyrOEt hydrolysis of C_1 esterase was approximately $1 \cdot 10^{-4}$ M. On the other hand, as shown in Table I, *p'*-GB-DBoG and *p'*-GB-DBiG inhibited C_1 esterase more than phenyl ϵ-guanidinocaproate and ϵ-GCA-CEP: the concentrations of *p'*-GB-DBoG and *p'*-GB-DBiG for 50% inhibition of C_1 esterase were $3.0 \cdot 10^{-5}$ and $3.4 \cdot 10^{-5}$ M, respectively.

Furthermore, *p'*-GB-DBiG inhibited $C_1\bar{r}$ more then C_1 esterase: the concentrations causing 50% inhibition of *N*-AcArgOMe hydrolysis by $C_1\bar{r}$ and *N*-AcTyrOEt hydrolysis by C_1 esterase were $4.4 \cdot 10^{-6}$ and $3.4 \cdot 10^{-5}$ M, respectively, as shown in Table I.

K_i values of these inhibitors on esterolysis using various substrates were then determined. Table II shows that the K_i value of ϵ-GCA-CEP for trypsin hydrolytic activity was $2.6 \cdot 10^{-6}$ M, the K_i value of *p'*-GB-DBoG was $8.1 \cdot 10^{-7}$ M, and K_i value of *p'*-GB-DBiG was $3.3 \cdot 10^{-8}$ M. K_i values of *p'*-GB-DBiG for plasmin, $C_1\bar{r}$, and C_1 esterase were $3.8 \cdot 10^{-8}$, $5.0 \cdot 10^{-6}$, and $1.2 \cdot 10^{-4}$ M, respectively. Modes of inhibitions by these compounds were all competitive. K_m values for trypsin, plasmin, $C_1\bar{r}$ and C_1 esterase were estimated as shown in

TABLE III

K_m VALUES FOR TRYPSIN, PLASMIN, $C_1\bar{r}$ AND C_1 ESTERASE

K_m values were estimated in 0.1 M sodium phosphate buffer, pH 7.4, at 37°C.

Enzyme	Substrate	K_m (M)
Trypsin	TosArgOMe	$3.1 \cdot 10^{-3}$
Plasmin	TosArgOMe	$6.0 \cdot 10^{-3}$
$C_1\bar{r}$	*N*-AcArgOMe	$1.2 \cdot 10^{-2}$
C_1 esterase	*N*-AcTyrOEt	$4.2 \cdot 10^{-2}$

TABLE IV

EFFECT OF DIALYSIS ON ESTEROLYSIS OF ε-GCA-CEP AND *p'*-GB-DBoG TREATED TRYPSIN

A mixture of trypsin (20 μg/ml) and inhibitor (10^{-3} M) in 0.1 M borate buffer containing 0.001 M $CaCl_2$, pH 8.5 was incubated at 37°C for 5 min and then dialyzed against the same buffer overnight, at 4°C. A mixture of 0.1 ml of dialyzate and 10 mM TosArgOMe were incubated at 37°C for 30 min and then the esterase activities of trypsin were determined. Values are shown in μmol per 0.1 ml.

	Buffer *	Inhibitor (10^{-3} M)	
		ε-GCA-CEP	*p'*-GB-DBoG
Dialysis (—)	19.94	3.62	2.36
Dialysis (+)	19.90	19.80	2.45

* 0.1 M borate buffer containing 0.001 M $CaCl_2$, pH 8.5

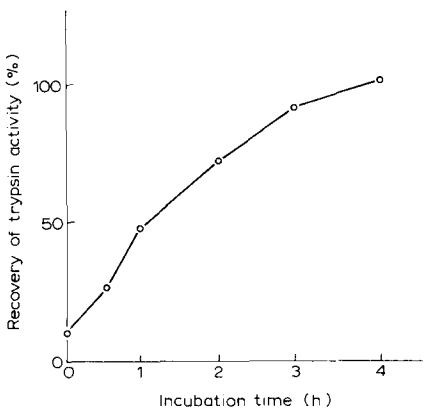


Fig. 2. Effect of incubation on esterolysis of the inhibitor · trypsin complex. A mixture of trypsin (20 μg/ml) and *p'*-GB-DBoG (10^{-3} M) in 0.1 M borate buffer containing 0.001 M $CaCl_2$, pH 8.5 was incubated at 37°C for 5 min and then dialyzed against the same buffer overnight, at 4°C. Amounts of 0.1 ml of dialyzate were incubated at 37°C for the indicated time intervals and esterase activities of trypsin were determined following incubation of the mixture at 37°C for 30 min after addition of 10 mM TosArgOMe.

Table III. The K_m value for trypsin with TosArgOMe as substrate was $3.1 \cdot 1\text{-}^{-3}$, $6.0 \cdot 10^{-3}$ M for plasmin with TosArgOMe, $1.2 \cdot 10^{-2}$ M for $C_1\bar{r}$ with *N*-AcArg-OMe, and $4.2 \cdot 10^{-2}$ M for C_1 esterase with *N*-AcTyrOEt.

The various compounds described above were reversible inhibitors of trypsin, plasmin, plasma kallikrein, thrombin, $C_1\bar{r}$ and C_1 esterase. For example, as shown in Table IV, ε-GCA-CEP was easily removed from the inhibited enzyme by dialysis. *p'*-GB-DBoG was not removed from the inhibited enzyme by dialysis, but after dialysis it was completely removed by incubating the inhibited enzyme at 37°C for 4 h, as shown in Fig. 2.

Experiments are now in progress on the mechanisms of inhibition of the various enzymes and the behaviors of these inhibitors.

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