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SYNTHETIC LOW MOLECULAR WEIGHT INHIBITORS OF SERUM KALLIKREIN

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Abstract—The influence of derivatives of benzylamine, benzamidine and phenylguanidine on the activity of serum kallikrein was investigated, and structure–activity relationships were derived. Among the substances tested, competitive, temporary and irreversible inhibitors of serum kallikrein were found. These three types of inhibitors are described and their possible use as effective inhibitors of kinin liberation *in vivo* is discussed.

IN THE COURSE of our studies on the chemical regulation of proteolytic reaction chains, such as the blood coagulation and fibrinolytic systems, we have investigated the possibility of influencing the kallikrein–kinin system in the blood. The liberation of kinins, pharmacologically highly active peptides, is also caused by proteolytic reaction chains. Kinin-liberating kallikreins exist in the blood and different organs.[1]

Recently serum kallikrein has received increasing attention, after it was found that under different pathological conditions an increase in activation of this enzyme from its precursor, kallikreinogen, occurs.[2,3] The special importance of serum kallikrein results from the fact that this enzyme exists in the bloodstream, while kinin-liberating enzymes from pancreas, salivary glands and kidney only act locally. Elucidation of the physiological and pathological role of the kallikrein-kinin system in the blood is complicated, because the organism possesses potent control mechanisms which inactivate the active serum kallikrein through kallikrein inhibitors present in the blood.[1] There are two ways of influencing the kallikrein-kinin system; first, the use of specific kinin antagonists which block the kinin receptors without any kinin action; second, the inhibition of kinin liberation by inhibition of the enzyme responsible for this liberation.

Attempts to develop specific kinin antagonists have not been successful. Another way was found by using naturally occurring kallikrein inhibitors of animal or vegetable origin which act by inhibiting the enzyme.[4–7] Up to now, the inhibitor from bovine organs is employed in practice. As a polypeptide this polyvalent inhibitor is effective only after parenteral application.[7] Therefore, it seemed worth while to look for other ways of inhibiting the action of kallikrein.

In our investigations on the pharmacological control of blood clotting and fibrinolysis it has been shown that benzamidine and benzylamine derivatives, which were found to be inhibitors of trypsin, thrombin and plasmin,[8–11] are inhibitors of serum kallikrein as well.[12–14]

In previous investigations we found that benzamidine and benzylamine derivatives are able to form adsorption complexes with the enzyme by ionic interaction because

of their similarity to basic amino acids. Moreover, some compounds were able to form more or less stable covalent bonds with the enzyme. Corresponding to the mechanism of action, there are three different types of inhibitors:[15] (1) Competitive inhibitors which form reversible complexes with the enzyme. The effectiveness of this type of inhibitors is characterized by its affinity to the enzyme. (2) Temporary inhibitors which react with the enzyme like substrates. After formation of the reversible complex, the active site of the enzyme is acylated and then deacylated. Substrates normally possess a high rate of acylation and deacylation of the active site of the enzyme. If the rate of deacylation is far slower than that of acylation, a temporary inhibition results. The potency of such inhibitors is not only influenced by their affinity but also by their rate of acylation and deacylation. (3) Irreversible inhibitors which are also covalently linked to the active site of the enzyme, but the acyl-enzyme formed in this way is stable and no deacylation takes place.

In the present paper the influence of these three types of inhibitors on the activity of serum kallikrein and structure–activity relationships with the enzyme were investigated. Moreover, we wanted to find out, which of these three types of inhibitors possesses optimal properties for chemical regulation of the kallikrein-kinin system *in vivo* in order to estimate their therapeutic use.

MATERIALS AND METHODS

Serum kallikrein was isolated from porcine serum according to Habermann and Klett.[16] The activity of the enzyme preparation was determined by active site titration with 4-nitrophenyl 4′-guanidinobenzoate according to Chase and Shaw.[17] It was found to contain 8×10^{-11} moles active enzyme per mg.

Kininogen in human serum obtained from platelet-free citrated plasma by recalcification in siliconized test tubes was used.

Bradykinin acetate 5 H_2O, synthetic, was obtained from Schering, Berlin.

Inhibitors. Benzylamine derivatives and different benzenesulfonylfluorides were synthesized by Kazmirowski. The synthesis is described elsewhere.[18–20] Benzamidine and guanidinobenzoic acid derivatives were synthesized by Wagner *et al.*[21,22]

Determination of serum kallikrein activity. The activity of serum kallikrein was determined by estimation of the amount of kinins liberated from human serum. The amount of kinins was determined by measuring their action on the isolated atropinized guinea pig ileum according to a method described earlier.[12]

To exclude the possibility that the inhibitors of kallikrein might have influenced the reactivity of the isolated guinea pig ileum bradykinin (0·05–0·15 μg) was added to the organ bath containing serum and the inhibitor. In our studies only those inhibitors were used which did not prevent the bradykinin-induced contraction of the ileum.

To estimate the inhibitory effectiveness of the competitive inhibitors the inhibitor concentration causing a 50 per cent inhibition was determined. Serum, 0·5 ml, was added to 9·5 ml of Tyrode's solution in the organ bath containing the inhibitor. In the control, the organ bath did not contain inhibitor. After addition of 0·05 ml (0·1 mg in Tris buffer 0·05 M, pH 7·2) of serum kallikrein solution, kinin liberation was initiated.

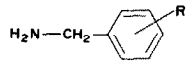
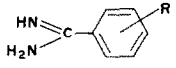
In the case of temporary and irreversible inhibitors 0·4 mg of the enzyme preparation were preincubated in 1 ml of inhibitor solution (in Tris buffer 0·05 M, pH 7·2).

As a control the enzyme was incubated in buffer solution without inhibitor. A sample containing 0·1 mg of the enzyme was taken from the incubation mixture and added to the organ bath which already contained 0·5 ml of serum.

RESULTS

Competitive inhibitors. Several derivatives of benzamidine and benzylamine were found to be inhibitors of serine proteinases.[8] The values of the inhibitory effect of the derivatives tested on serum kallikrein are shown in Table 1. Most of the benzamidine derivatives inhibited serum kallikrein more strongly than the corresponding benzylamine derivatives. Benzylamine (I) did not inhibit the enzyme at a concentration of 5×10^{-3} M. More effective inhibitors were obtained by halogenation, especially in the case of 3,4-dichlorbenzylamine (XI) or by combination with an aromatic component (VII).

TABLE 1. INHIBITION OF SERUM KALLIKREIN BY DERIVATIVES OF BENZYLAMINE AND BENZAMIDINE

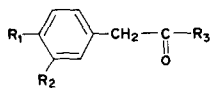
	R	Position	 I_{50}(mM)	 I_{50}(mM)
I	—H		>5·0	1·0
II	—NH_2	4	>5·0	0·15
III	—NH—CO—CH_3	4	>5·0	0·6
IV	—CH_2—NH_2	4	1·0	0·03
V	—O—CH_3	4	3·0	1·0
VI	—O—CH_3	3	>5·0	1·0
VII	—O—CH_2— 	4	0·03	0·1
VIII	—CH_3	4	1·0	0·5
IX	—Cl	4	1·0	0·7
X	—Cl	3	1·0	0·8
XI	—Cl, —Cl	3, 4	0·15	0·5
XII	—CH_2—CO—COOH	4	0·3	0·006

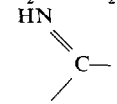
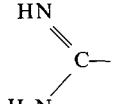
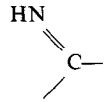
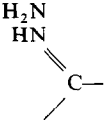
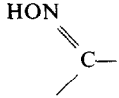
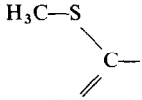
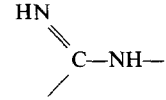
Benzamidine (I) was much more effective than benzylamine. Structure–activity relationships, as far as they could be derived from the benzylamine derivatives tested, were not always comparable to those of the corresponding benzamidine derivatives.

Significant differences could be shown in the case of amino derivatives of benzylamine and benzamidine (II), respectively. 4-Aminomethylbenzamidine (IV) which can be considered either as a benzamidine or as a benzylamine derivative was found to be especially effective. It is evident that among the benzylamine and benzamidine derivatives tested, 4-amidinophenylpyruvic acid (XII) is the most potent inhibitor of serum kallikrein. The corresponding benzylamine derivative, 4-aminomethylphenylpyruvic acid (XII), exerted only a small influence on the kinin-liberating activity of this enzyme. Esterification of 4-amidinophenylpyruvic acid to 4-amidinophenylpyruvic acid ethyl ester (XVII) did not result in a further increase in inhibitory activity.

To elucidate the influence of the basic group on the affinity of the inhibitor, chemical structure and position of this group were modified. The results are shown in Table 2.

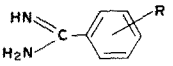
TABLE 2. INHIBITION OF SERUM KALLIKREIN BY DERIVATIVES OF PHENYLPYRUVIC ACID

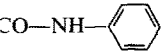
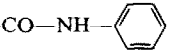
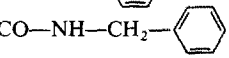


	R_1	R_2	R_3	I_{50}(mM)
XIII	H_2N—	H—	—COOH	>1·0
XIV	H—	H_2N—	—COOH	>1·0
XV	H_2N—CH_2—	H—	—COOH	>1·0
XVI		H—	—COOH	0·006
XVII		H—	—$COOC_2H_5$	0·005
XVIII	H—		—COOH	0·3
XIX	H—		—$COOC_2H_5$	0·005
XX		H—	—COOH	1·1
XXI		H—	—COOH	>1·0
XXII		H—	—COOH	>1·0

Replacement of the amidino moiety with an amino (XIII) or guanidino group (XXII) led to a significant fall in potency. By substitution of one of the nitrogen atoms of the amidino group (XXI) the inhibitory activity was also decreased. Derivatives of phenylpyruvic acid with an amino or amidino group at the 3 position of the ring (XIV, XVIII) were less effective than 4-amidinophenylpyruvic acid. However, esterification of 3-amidinophenylpyruvic acid (XIX) resulted in a significant increase in inhibitory activity. This inhibitor was found to have about the same activity as 4-amidinophenylpyruvic acid. Compounds related to amidinophenylpyruvic acid possess only weak inhibitory potency, for example 3-amidinocinnamic acid (XXVII) and 4-ami-

TABLE 3. INHIBITION OF SERUM KALLIKREIN BY DERIVATIVES OF AMIDINOPHENYLCARBOXYLIC ACID



	R	Position	I_{50}(mM)
XXIII	$-CH_2-CH_2-COOH$	4	>1·0
XXIV	$-CH_2-CH_2-COOCH_3$	4	0·02
XXV	$-CH_2-CH_2-COOCH_3$	3	0·5
XXVI	$-CH=CH-COOC_2H_5$	4	0·34
XXVII	$-CH=CH-COOH$	3	0·2
XXVIII	$-CH=CH-COOC_2H_5$	3	0·04
XXIX	$-CO-NH_2$	4	0·2
XXX	$-CO-NH-CH_3$	4	1·0
XXXI		4	0·1
XXXII		3	0·2
XXXIII		4	0·2

dinophenylpropionic acid (XXIII). Esterification of 4-amidinophenylpropionic acid (XXIV) led to an increase in inhibitory activity (Table 3).

Temporary inhibitors. According to the definition given above variously substituted phenyl amidinobenzoates, phenyl guanidinobenzoates and amidinophenyl benzoates were found to be temporarily acting inhibitors.[11,23] As a consequence of the mechanism of action of these compounds the inhibition continued after dilution in the organ bath. After different periods of preincubation a time-dependent inhibition of the enzyme resulted, rising to a maximum and decreasing after a long period of incubation (Figs 1, 2). In contrast to the aromatic esters of guanidinobenzoic acid, the aromatic amidinobenzoates tested showed a slow rate of acylation.

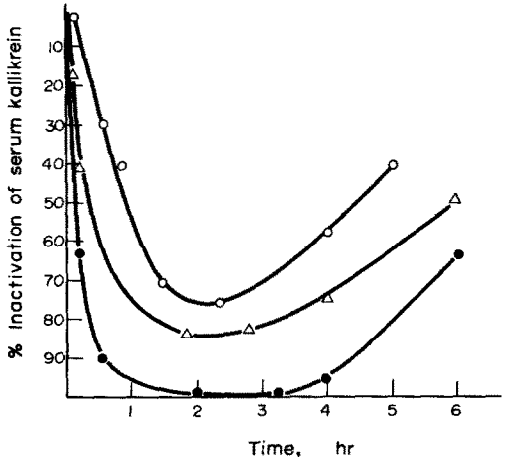


FIG. 1. Inactivation of serum kallikrein (3×10^{-8} M in 0·05 M Tris-HCl buffer) by 4-amidinophenyl benzoate at pH 7·2 and 25°. (●) 5×10^{-7} M; (△) 1×10^{-7} M; (○) 5×10^{-8} M.

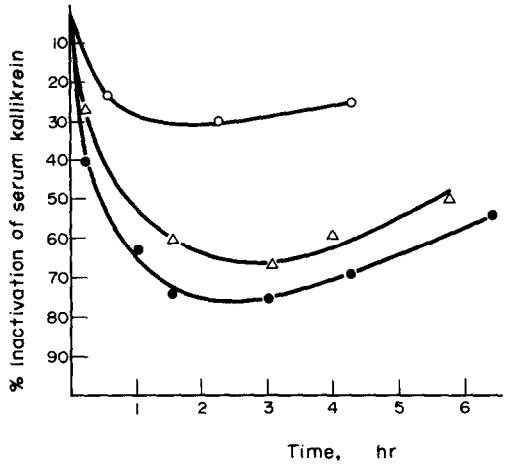
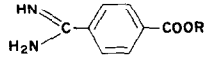
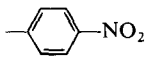
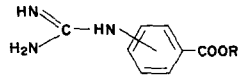


FIG. 2. Inactivation of serum kallikrein (3×10^{-8} M in 0·05 M Tris-HCl buffer) by phenyl 4-guanidinobenzoate at pH 7·2 and 25°. (●) 5×10^{-7} M; (△) 1×10^{-7} M; (○) 5×10^{-8} M.

TABLE 4. INACTIVATION OF SERUM KALLIKREIN BY ESTERS OF AMIDINO- AND GUANIDINOBENZOIC ACIDS



	R	I_{50} (mM) after 10 min incubation
XXXIV		0·2
XXXV		0·2



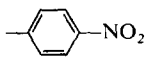
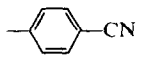
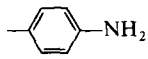
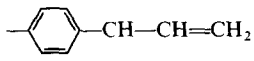
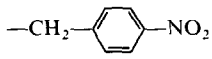
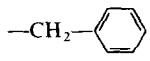
	R	Position of the guanidino group	I_{50} (mM) after 10 min incubation
XXXVI		3	>0·1
		4	0·0005
XXXVII		3	>0·1
		4	0·001
XXXVIII		4	0·05
XXXIX		4	0·01
XL		4	0·05
XLI		3	>0·1
		4	>0·1
XLII		3	>0·1
		4	0·1

TABLE 4 (contd)

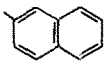
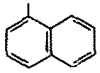
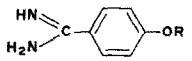
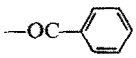
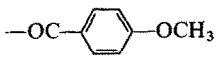
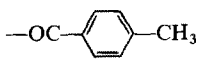
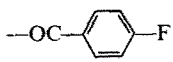
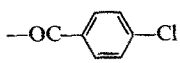
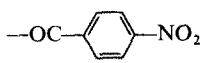
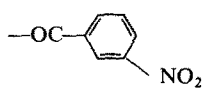
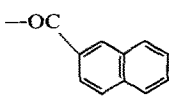
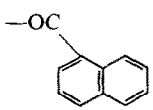
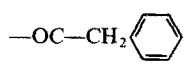
	R	Position of the guanidino group	I_{50} (mM) after 10 min incubation
XLIII		3	>0·1
		4	0·008
XLIV		3	>0·1
		4	0·065

TABLE 5. INACTIVATION OF SERUM KALLIKREIN BY DERIVATES OF 4-AMIDINOPHENYL BENZOATE



	R	I_{50} (mM) after 10 min incubation
XLV		0·0004
XLVI		0·005
XLVII		0·0003
XLVIII		0·001
IL		0·001
L		0·005
LI		0·01
LII		0·0009
LIII		0·0015
LIV		0·005

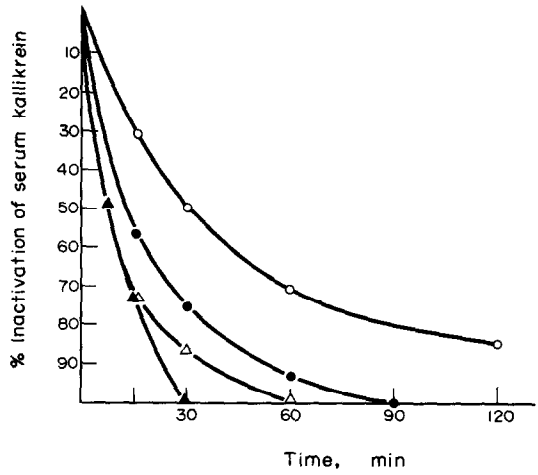
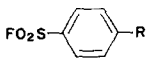


FIG. 3. Inactivation of serum kallikrein by aminoalkyl benzenesulfonyl fluorides (5×10^{-4} M) at pH 7·2 and 25°. (▲) 4-aminomethyl benzenesulfonyl fluoride; (△) 4-amidino benzenesulfonyl fluoride; (●) 4-(1-aminoethyl) benzenesulfonyl fluoride; (○) 4-(2-aminoethyl) benzenesulfonyl fluoride.

To compare the inhibitory effect of different substituted esters the concentration causing a 50 per cent inhibition (I_{50}) of the enzyme after 10 min of incubation was determined. These values are not representative of the course of inhibition, they serve for comparison only. Results are shown in Table 4. Under these conditions, 4-nitrophenyl 4′-guanidinobenzoate (XXXVI) and phenyl guanidinobenzoate (XXXVII) were found to be the most potent inhibitors. Amidinophenyl benzoates were shown to be highly effective as well (Table 5).

Irreversible inhibitors. In previous investigations it was shown that competitive inhibitors combined with chemically reactive groups are transformed into inhibitors

TABLE 6. RATE CONSTANTS FOR THE INACTIVATION OF SERUM KALLIKREIN BY BENZENESULFONYL FLUORIDES



	R	Constant of inactivation k (M^{-1} sec^{-1})
LV	$-NH_2$	<0·1
LVI	$-NH-CO-CH_3$	<0·1
LVII	$-CH_2-NH_2$	3·44
LVIII	$-CH_2-NH-CO-CH_3$	0·28
LIX	$-CH(CH_3)-NH_2$	1·22
LX	$-CH_2-CH_2-NH_2$	0·68
LXI	$-CH_2-CH_2-NHCOCH_3$	0·21
LXII	$-C(NH_2)=NH$	2·78
LXIII	Phenylmethylsulfonyl-fluoride (PMSF)	0·07
LXIV	Diisopropylfluoro-phosphate (DFP)	0·3

which form a stable covalent bond with the enzyme.[24,25] By this reaction the enzyme is irreversibly blocked. Starting from the competitive inhibitors benzylamine and benzamidine, we studied the kallikrein-inactivating action of derivatives of these compounds with a reactive sulfonyl fluoride moiety on the aromatic ring. Inactivation of serum kallikrein occurred in a time-dependent reaction (Fig. 3). The inactivation of serum kallikrein by aminoalkyl benzenesulfonyl fluorides occurred via a bimolecular reaction. The inhibitory potency of these compounds was determined by measuring the second order rate constant k (M^{-1} sec^{-1}).

Among the compounds tested aminomethyl (LVII) and amidino benzenesulfonyl fluoride (LXII) caused the highest rate of inactivation (Table 6). If, starting from the highly effective aminomethyl benzenesulfonyl fluoride, the side chain was prolonged resulting in a greater distance of the amino group from the aromatic ring, the inhibitory potency decreased again (LX). Acetylation of the amino group caused a diminution of the inhibitory effect. The aniline derivative, 4-amino benzenesulfonyl fluoride (LV), showed only a weak inactivating effect. Under physiological conditions, the ring substituted amino group of this compound is considerably less protonated. The inhibitory effect of both aminoalkyl benzenesulfonyl fluorides and amidino benzenesulfonyl fluorides surpassed that of diisopropylfluorophosphate (LXIV) and phenylmethylsulfonyl fluoride (LXIII) which are commonly used as irreversible inhibitors of serine proteinases.

DISCUSSION

Our investigations were performed with the aim of developing synthetic kallikrein inhibitors which might be used *in vivo* for therapeutic purposes. To estimate the possible therapeutic use of these inhibitors, their behaviour in the blood and *in vivo* had to be clarified.

Since the enzyme spontaeously activates and reacts with a substrate which is permanently available, only those inhibitors are effective that interfere immediately with this reaction.

Stimulated by the considerable antikallikrein action of different temporarily acting inhibitors, we studied their activity in the blood. These inhibitors must possess a high rate of acylation and a low rate of deacylation besides having a high affinity to the enzyme. Aromatic esters of guanidinobenzoic acid meet these requirements. However, further studies revealed that these esters are hydrolized in the blood.[26] After incubation of the ester with serum the inhibitory action of phenylguanidinobenzoate on the kinin-liberating activity of serum kallikrein was abolished. Irreversibly acting inhibitors, such as benzenesulfonyl fluorides, also are unable to prevent serum kallikrein-induced kinin liberation in blood, because these compounds acylate the enzyme too slowly. Therefore, it is not likely that they will be useful in therapy.

In earlier investigations we showed that the drop in blood pressure in rabbits caused by injection of serum kallikrein could be prevented by application of the competitive inhibitor 4-amidinophehylpyruvic acid.[13] Glass contact-induced kinin liberation in serum was also inhibited by addition of competitive inhibitors. These experiments demonstrate in principle the possibility of a use of competitive synthetic inhibitors *in vivo*.

In connection with these investigations we should like to comment on the work of Davis and Lowe.[27] Geratz[28,29] and Geratz and Webster.[30] According to these authors, *bis*amidino derivatives were found to be potent competitive inhibitors of esterolytic and kinin-liberating activity of guinea pig serum kallikrein and porcine pancreatic kallikrein. However, *in vivo bis*amidines provoked a marked decrease in blood pressure as a side effect. Histamine liberation as well as adrenergic blockade caused by these compounds are discussed in this respect.[30] These side effects prevent the therapeutic use of these compounds.

The structure–activity relationships found in our investigations on serum kallikrein generally agree with those found for other serine proteinases.[8,10,11,25] This is not surprising, since kallikrein belongs to the category of serine proteinases which can be inactivated by diisopropylfluorophosphate.[31,32] Because of its side chain specificity it possesses strong similarity to trypsin and trypsin-like enzymes. Thus, at present no specific inhibitor of serum kallikrein exists.

Besides their possible therapeutic use, further experiments on synthetic low molecular inhibitors of serum kallikrein should lead to an elucidation of the role of the kallikrein-kinin system in physiological and pathological processes.

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