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INHIBITION OF THE HAEMOLYTIC COMPLEMENT ACTIVITY BY DERIVATIVES OF BENZAMIDINE

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Abstract—The influence of a series of benzamidine derivatives on the haemolytic complement activity is studied. Complement-mediated haemolysis is inhibited by the derivatives tested to a different extent. Aromatic diamidines are found to be relatively potent inhibitors. Hydrophobic substituents of benzamidine are believed to enhance the inhibitory activity. Relations to the inhibitory action of benzamidine derivatives on serine proteinases of the blood are discussed.

The complement system of the sera of mammals consists of eleven distinct components representing proteins or glycoproteins [1]. In the process of immunological reactions activation of the complement system takes place through immune-complexes or antibody-coated cells leading to phenomena, such as immune adherence, cell lysis, phagocytosis and chemotaxis [2]. Moreover, in the course of complement activation peptides are released that act as inflammation mediators. Activation proceeds in a cascade-like sequence of reactions, in which specific proteolytic processes play an essential role [2]. The complement system shows analogies in structure and activation mechanism to the clotting, fibrinolytic and kinin forming systems [3]. Furthermore, interrelationships exist between the proteolytic enzyme systems which let one suppose that pathological activation of one of the particular systems mentioned brings about activation of the complement system [4–6].

Though the role of complement in a number of "immune complex diseases" has not yet been fully clarified, it seems feasible to influence immune reactions by chemical control of complement activity. The naturally occurring inhibitors of complement components such as the $C\bar{1}$ inhibitor are proteins. Therefore, their therapeutic use is limited. Since enzymatically active subunits of the first complement component were shown to be serine proteinases with trypsin-like substrate specificity [7, 8], it seemed worthwhile to investigate synthetic inhibitors of serine proteinases developed by us for their possible anticomplement activity. From a variety of synthetic competitive inhibitors derivatives of benzamidine were selected and their influence on complement-mediated lysis of sensitized erythrocytes was studied. The results are compared to those obtained in other enzyme systems [9–15].

MATERIALS AND METHODS

Erythrocytes. Suspension of sheep erythrocytes in Alsever's solution.

Haemolysin. Rabbit antibody against sheep erythrocytes; VEB Sächsisches Serumwerk Dresden, GDR.

Complement. Guinea pig serum, prepared from sterile withdrawn blood, pooled and deep frozen.

Buffer. Tris–sodium chloride–gelatin buffer pH 7.4 (5 mM Tris, 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, 0.15 M NaCl, 0.1% gelatin). Citrate–saline solution: 56.3 mM sodium citrate, 37.5 mM sodium chloride.

Pentamidine. Lomidine, SPECIA, Paris, France.

Benzamidine derivatives were synthetized as described previously and had been already tested in other enzyme systems [9–15].

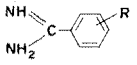
Measurement of haemolytic complement activity was performed by modification of the method of Baker and Erickson [16]: erythrocytes were separated from Alsever's solution by centrifugation and washing. A suspension of erythrocytes in buffer (1×10^9 cells/ml) was incubated with haemolysin (1:800 diluted in buffer) for 15 min at 37°. Excess haemolysin was removed by washing the cells twice. For the reaction of sensitized erythrocytes with complement 0.5 ml erythrocyte suspension (1×10^9 cells/ml), 0.2 ml guinea pig serum (1:32 diluted in buffer), 0.2 ml buffer and 0.1 ml inhibitor (in buffer containing 10% methanol) or 0.1 ml buffer alone were mixed. Guinea pig serum and inhibitor were added subsequently. After incubation at 37° for 4 min the reaction was stopped by adding 2.0 ml of ice-cold citrate–saline solution. Cell-free supernatant was obtained by centrifugation at 2000 *g* for 10 min at 4°. The extinction of the supernatant was measured at 541 nm in a spectrophotometer (VSU 1, Carl Zeiss Jena).

Inhibitor-free controls with varying complement activities from which standard curves were obtained run parallel. Pentamidine was used as reference inhibitor in order to evaluate differences in sensitivity of the different cell batches. The benzamidine derivatives were tested for possible haemolytic effects in complement-free controls. Controls for spontaneous haemolysis contained neither complement nor inhibitor. From standard curves (extinction versus complement activity) percentages of inhibition were calculated for each concentration of benzamidine derivatives used. That concentration causing 50 per cent inhibition of complement activity (I_{50}-value in mM) was graphically estimated by lin–log plots (per cent inhibition vs concentration).

RESULTS

The inhibitory activity of the substances tested (Table 1: amidinophenyl ethers, Table 2: amidinophenyl ketones, Table 3: aromatic diamidines) is

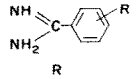
Table 1. Inhibition of haemolytic complement by benzamidine, *p*-chlorobenzamidine, *p*-aminobenzamidine and amidinophenyl ether derivatives



	R	Position	t_{50} (mM)
I	H	4	4.7
II	Cl	4	>5.0
III	NH_2	4	0.46
IV	$O-C_2H_5$	4	4.2
V	$O-C_4H_9$	4	4.2
VI	$O-C_5H_{11}$	4	2.6
VII	$O-C_5H_{11}$	3	1.3
VIII	$O-C_7H_{15}$	4	0.5*
IX	$O-C_7H_{15}$	3	0.3
X	$O-C_8H_{17}$	4	0.3*
XI	$O-C_{10}H_{21}$	4	0.1*
XII	$O-C_{10}H_{20}OH$	4	1.0
XIII	$O-CH_2-C_6H_5$	4	5.3
XIV	$O-CH_2-C_6H_5$	3	2.5
XV	$O-C_3H_6-C_6H_5$	4	1.8
XVI	$O-C_3H_6-C_6H_5$	3	1.0

* Extrapolated values

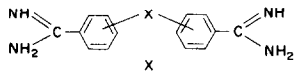
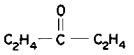
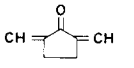
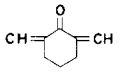
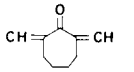
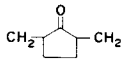
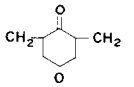
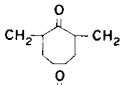
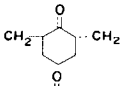
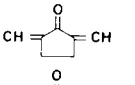
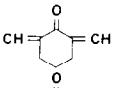
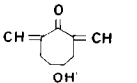
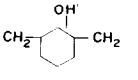
Table 2. Inhibition of haemolytic complement by amidinophenyl ketone derivatives



	R	Position	t_{50} (mM)
XVII	$CH_2-C(=O)-CH_2OH$	4	>5.0
XVIII	$CH_2-C(=O)-COOH$	4	0.43
XIX	$CH_2-C(=O)-COOH$	3	1.3
XX	$CH_2-C(=O)-C_6H_5$	4	2.8
XXI	$CH_2-C(=O)-C_6H_4-Cl$	4	1.5
XXII	$CH_2-C(=O)-C_{10}H_7$	4	0.48
XXIII	$CH_2-C(=O)-CH_2-C_6H_5$	4	>5.0
XXIV	$CH_2-C(=O)-CH_2-O-C(=O)-C_6H_5$	4	2.5
XXV	$CH_2-C(=O)-CH_2-O-C(=O)-C_{10}H_7$	4	1.5*
XXVI	$CH_2-CHBr-C(=O)-C_6H_5$	4	>5.0
XXVII	$CH_2-CH_2-C(=O)-C_{10}H_7$	4	0.7

* Extrapolated value

Table 3. Inhibition of haemolytic complement by aromatic diamidines

	 X	Position	I_{50} (mM)
XXVIII	$O-C_5H_{10}-O$	4,4′	0.51
XXIX	CH_2-O	4,4′	2.40
XXX	CH_2-O	4,3′	1.30
XXXI	CH_2-O	3,3′	0.70
XXXII		4,4′	0.70
XXXIII		4,4′	0.42
XXXIV		4,4′	0.20
XXXV		4,4′	0.38
XXXVI		4,4′	0.31
XXXVII		4,4′	0.37
XXXVIII		4,4′	0.31
XXXIX		4,4′	0.75
XL		3,3′	0.07
XLI		3,3′	0.25
XLII		3,3′	0.24
XLIII		4,4′	0.26

expressed as I_{50}-values. Benzamidine (I) and its *p*-chloro derivative (II) are weak inhibitors, whereas *p*-amino benzamidine (III) possesses considerably higher activity (I_{50} = 0.46 mM).

The alkoxy derivatives (IV to XII) show an increasing activity with increasing length of the side chain; for the substances VIII to XI only extrapolated I_{50}-values can be given because of the haemolytic effect of some concentrations used. The aralkoxy derivatives XV and XVI possess higher inhibitory activity than benzamidine, the increased activity might be caused by the stronger lipophilic nature of the substituents.

The I_{50}-values of the derivatives with a keto group (Table 2) are in the same range as that of benzamidine. *p*-Amidinophenyl pyruvic acid (APPA (XVIII)) exerts a relatively strong effect (I_{50} = 0.43 mM). The activity of APPA is reached by the substances XXII and XXVII only. Also in this group of derivatives an increase in activity with stronger lipophilic nature of the substituents is seen.

The aromatic diamidines (Table 3), as a whole, possess stronger inhibitory activities than the derivatives with one amidino group. Pentamidine has an activity one order of magnitude higher than that of benzamidine (I_{50} = 0.51 mM). The substances XXIX to XXXII show weaker activities. The diamidines with a cyclic keto group (XXXIII to XLII) are relatively potent inhibitors of the haemolytic complement action. A remarkably strong activity exerts substance XL, with an I_{50}-value of 0.07 mM.

In all groups of benzamidine derivatives the meta-isomers are more active than the corresponding para-derivatives.

The dose–response curves (per cent inhibition of complement activity vs inhibitor concentration) revealed considerable differences for certain substances, e.g. substance XL has a flat slope of the curve, whereas the curves of APPA and pentamidine are steeper (Fig. 1).

DISCUSSION

Investigations on enzymatic activities of the complement system have shown that subunits C1r and C1s of the first complement component are serine

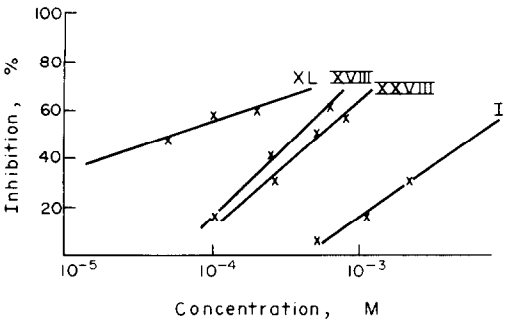


Fig. 1. Dose–response curves of some benzamidine derivatives tested. (Per cent inhibition of complement calculated from standard curves. For details see "Materials and Methods", for chemical structure see Tables 1–3.)

proteinases [7, 8, 17, 18]. C1 esterase hydrolyses synthetic substrates such as TAME and ATEE and is inhibited by DFP and other organophosphorous compounds. Moreover, certain derivatives of aromatic amino acids, ω-amino carbonic acids as well as mono and diaminoalkanes inhibit C1 esterase [19–23]. Derivatives of benzamidine also exert inhibitory influence on C1 esterase and haemolytic complement activity [16, 24, 25]. Benzamidine derivatives with a terminal SO_2F-group are irreversible inhibitors of C1 esterase [26, 27]. Furthermore, the inhibitory effect of aromatic diamidines on the enzymatically active subcomponents of $C\bar{1}$, namely C1r and $C\bar{1}s$, and on the generation of C1 esterase is described [28]. The benzamidine derivatives studied are competitive inhibitors of the serine proteinases trypsin, thrombin, plasmin, and serum kallikrein [9–15]. They were also found to be inhibitors of the complement-mediated lysis of sensitized erythrocytes. To inhibit this reaction higher concentrations of benzamidine derivatives were required than for the inhibition of other reactions triggered by serine proteinases, like blood clotting, fibrinolysis and kinin generation.

Among the benzamidine derivatives tested, compounds with substituents in meta-position were more effective than those with substituents in para-position. This difference in activity between the position-isomers was also reported by Baker and coworkers [16, 24, 25]. The most potent inhibitors were found in the group of aromatic diamidines. Similar concentrations of aromatic diamidines, as they are effective in inhibiting C1 esterase in isolated system, also inhibit immune haemolysis.

In quantitative analyses of structure–activity relationships on a number of benzamidine derivatives tested by the Baker group, Coats found a correlation between the inhibitory activity on thrombin and the activity on haemolytic complement [29]. Hansch and Yoshimoto concluded from their analyses that a second aromatic nucleus of benzamidine derivatives linked to the benzamidine nucleus through a carbohydrate bridge contributes considerably to inhibitory activity [30]. However, in both analyses mainly *m*-substituted benzamidine derivatives were evaluated.

Our results confirm the assumption that lipophilic substitution of benzamidine is essential for a strong inhibitory activity on haemolytic complement. Similar relations exist for the serine proteinases trypsin, thrombin, and plasmin, in which a second hydrophobic binding site in the vicinity of the active centre is assumed to participate in binding of lipophilic benzamidine derivatives. The compounds tested show, with little exception, a parellelism between the values of inhibition of thrombin [9–15] and those of inhibition of haemolytic complement.

Considerable differences were shown to exist between inhibition of the C1 esterase and inhibition of haemolytic complement activity [31]. In order to explain these differences besides nonspecific binding of benzamidine derivatives in more complex test systems additional effects have to be taken into account. Activation of the complement sequence may be inhibited, inhibition of the C3 and C5 converting enzymes may occur, and the reaction of complement components with natural inhibitors may be influenced. The various mechanisms of complement activation, especially via the alternate pathway (for review see Osler and Sandberg [32]), allow the assumption that inhibition at the site of $C\bar{1}$ is only one mode of chemical control of complement activity. Elucidation of the mechanism of complement components C3 to C9 might lead to further possibilities of chemical control of the complement system.

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