Inhibition of Staphylococcal α -Toxin

A KINETIC EVALUATION OF AROMATIC POLYSULPHONIC ACIDS AS INHIBITORS OF HAEMOLYSIS

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1. The effect of a number of aromatic polysulphonic acids on the kinetics of haemolysis of rabbit erythrocyte suspensions by crude staphylococcal α -toxin was studied at pH 8.6 and 6.8. 2. All of the inhibitory compounds caused an increase in the prelytic lag time (τ) of the sigmoid haemolysis curves, an increase in the time to reach 50% haemolysis (t_1) and a decrease in the maximum rate of haemolysis ($R_{\max}$). The most inhibitory compounds caused a 50% decrease in $R_{\max}$ at concentrations between 0.1 and 0.2 mM. 3. The effect of pH varied considerably: compounds (I) and (II) were almost equally inhibitory at both pH values, compounds (IV) and (IX) were more inhibitory at pH 6.8 than at pH 8.6, and compounds (VII), (VIII), (X), (XI) and (XII) were more inhibitory at pH 8.6. 4. Increased time of premixing α -toxin with compound (I) caused increased inhibition. 5. An attempt was made, where possible, to relate the inhibitory activity to the structure of the test compound.

The α -toxin of *Staphylococcus aureus* is a potent haemolytic and lethal exotoxin produced by almost all pathogenic staphylococci. It is a protein that can exist in different molecular aggregates (Arbuthnott, Freer & Bernheimer, 1967); the biologically active form has a sedimentation coefficient of approx. 3s (Bernheimer & Schwartz, 1963; Lominski, Arbuthnott & Spence, 1963; Cooper, Madoff & Weinstein, 1966; Coulter, 1966). The physical, chemical and biological properties of α -toxin have been reviewed in detail by Bernheimer (1965) and Gladstone (1966). Although the kinetics of the haemolytic reaction are not inconsistent with the toxin's being an enzyme (Lominski & Arbuthnott, 1962; Marucci, 1963; Cooper, Madoff & Weinstein, 1964a), the substrate or receptor in cell membranes has not been identified. It has been suggested that α -toxin may react with artificial lipid spherules by means of an interaction between a hydrophobic portion of the toxin molecule and the constituent lipids (Weissmann, Sessa & Bernheimer, 1966).

The inhibition of staphylococcal α -toxin by chemical agents has not been extensively investigated. Sucrose and polyethylene glycol, when used at high concentrations, inhibit haemolysis by osmotically stabilizing erythrocytes damaged by α -toxin (Cooper, Madoff & Weinstein, 1964b). North & Doery (1961) described the inhibition of

the lethal effect of α -toxin by cerebrosides, but the mechanism of this inhibition is not clear.

The present study showed that the well-known enzyme inhibitor suramin (Wills & Wormall, 1950; Mills, Paul & Smith, 1953; Lominski & Gray, 1961), which is a naphthalenesulphonic acid, inhibited both the lethal and the haemolytic action of α -toxin. In an attempt to obtain more potent inhibitors for use in studying the inhibition of the lethal effect of α -toxin *in vivo* and to investigate the mechanism of inhibition, analogues of suramin and related compounds were compared by examining their effects on the kinetics of haemolysis. A collateral study of the inhibition of the lethal effects of α -toxin in mice is presented in the following paper (Arbuthnott, Lominski & Robson Wright, 1968).

Crude preparations of α -toxin from *Staphylococcus aureus*, strain Wood 46, the classical α -toxin-producing strain, were used; at least 90% of the haemolytic activity of such preparations is due to α -toxin and is neutralizable by specific antitoxin. Results obtained with crude toxin may not apply in full to pure toxin; however, crude toxin can be prepared in large quantities, and it provides a stable preparation suitable for the screening of a large number of test compounds under various conditions. It was hoped that such an investigation would allow the selection of compounds suitable

for a detailed investigation of the interaction between pure α -toxin and aromatic polysulphonic acids.

MATERIALS AND METHODS

Production and assay of crude staphylococcal α -toxin. Crude α -toxin was prepared from cultures of *Staphylococcus*

aureus, strain Wood 46 (N.C.T.C. 7121), grown on semi-solid nutrient agar in the presence of 20% CO₂ (Lominski & Arbuthnott, 1962). The supernatant fluid obtained after the cells had been centrifuged was freeze-dried in 0.5 ml. amounts and stored at -20°. Estimation of haemolytic activity by titration against washed rabbit erythrocytes was carried out as described by Lominski & Arbuthnott

Group 2

Group 3

Scheme 1. Compounds tested as possible inhibitors of staphylococcal α -toxin.

(1962); such preparations contained 16000 minimal haemolytic doses (M.H.D.)/ml.

Determination of inhibitory activity. A turbidimetric

method similar to that of Turpin, Relyfeldt, Pillet & Raynaud (1954) was used to measure the rate of haemolysis of suspensions of rabbit erythrocytes by a constant amount

of α -toxin (20 M.H.D.) in the presence of various concentrations of test compounds at 25°. All reagents were dissolved or suspended in iso-osmotic phosphate-buffered saline composed of 0.6% NaCl in 50mm-sodium phosphate buffer (Hendry, 1948). The decrease in extinction at 650m μ was followed with time in a Unicam SP.600 spectrophotometer in cuvettes of 1cm. light-path. A dilute suspension (0.2%) of rabbit erythrocytes was employed in the assay, since the relationship between extinction and erythrocyte concentration was linear under these conditions.

Reaction mixtures (total volume 3ml.) contained 1.5ml. of phosphate-buffered saline at the desired pH, 0.5ml. of a dilution of α -toxin (20 M.H.D.), 0.5ml. of a solution of test compound and 0.5ml. of a 0.2% (v/v) suspension of rabbit erythrocytes. The erythrocyte suspension was added immediately after thorough mixing of the other components of the reaction mixture. Extinctions were measured against a blank containing phosphate-buffered saline and test compound, at 15sec. intervals, beginning 45sec. after the addition of the erythrocyte suspension. Control runs were carried out in each experiment with the same concentration of α -toxin but in the absence of test compound. At least two kinetic runs were carried out for each concentration of test compound. Experiments were carried out at pH 8.6 and 6.8. At pH 6.8 a slight increase in extinction was observed during the prelytic lag phase, possibly due to a change in size or shape and light-scatter of the erythrocytes before lysis; this did not occur at pH 8.6. Most experiments were carried out at pH 8.6, but to test the effects of pH on inhibition the most effective inhibitors were tested also at pH 6.8.

Three criteria were used to assess the effectiveness of test compounds as inhibitors: (1) the lag time (τ), or time (min.) before the onset of haemolysis, which could be measured with a reproducibility of ± 0.5 min.; (2) the time (min.) required to reach 50% haemolysis (t_1), which could also be measured with a reproducibility of ± 0.5 min.; (3) the value of the quotient $R_{max.}$, which is the maximum rate of haemolysis divided by the maximum extinction of the erythrocyte suspension at 650m μ ; the denominator corrects for small differences in extinction of different erythrocyte suspensions. The maximum rate of haemolysis here refers to a maximum rate for any one run, i.e. the maximum absolute value taken by the slope of the plot of extinction against time, the slope being estimated by drawing tangents to the curve.

Test compounds. The structures of the compounds tested are shown in Scheme 1. Compounds (II), (IV) and (V) were kindly supplied by Dr K. R. Koch, Fabersfabriken Bayer A.-G., Pharma Wiss. Med. Entwicklung, Leverkusen, Germany, and the remainder by Dr F. L. Rose and Dr D. Case, Imperial Chemical Industries Ltd., Pharmaceutical Division, Alderley Park, Macclesfield, Cheshire.

Compound (V) could not be studied at pH 8.6 and compound (VI) at pH 6.8 because they were haemolytic at these values; each could, however, be studied at the alternative pH. Compounds containing the dichlorotriazine ring were susceptible to hydrolysis of one of the chlorine atoms, yielding the corresponding monohydroxy compound. This was most apparent with compound (XI), which was rapidly hydrolysed at both pH values. Solutions of these compounds were therefore prepared immediately before being added to the reaction mixture. Some hydrolysis, however, probably occurred during the experiment.

Compound (XII) was oxidized in solution on standing and the solution of it too was prepared immediately before use.

Rabbit erythrocytes. Rabbit blood was obtained by bleeding from the ear vein into 3.8% (w/v) trisodium citrate to prevent clotting. The same rabbit was used throughout as donor. The erythrocytes were washed at least four times in iso-osmotic phosphate-buffered saline of the desired pH and stored at 4°; stock preparations were discarded after 5 days. A 0.2% suspension of packed erythrocytes was prepared for use in kinetic experiments.

RESULTS

The effect of increasing concentrations of compound (VIII) on the kinetics of haemolysis by staphylococcal α -toxin at pH 8.6 is shown in Fig. 1. Essentially similar curves were obtained for all the compounds that proved to be inhibitory. Both the lag time (τ) and the time to reach 50% haemolysis (t_1) increased with increasing concentrations of test compound, whereas the rate of haemolysis ($R_{max.}$) decreased. The relationship between these different kinetic criteria and the concentration of

Fig. 1. Effect of concentration of compound (VIII) on haemolysis of rabbit erythrocytes by staphylococcal α -toxin. To 1.5ml. of iso-osmotic phosphate-buffered saline were added 0.5ml. of α -toxin dilution (20 M.H.D.), 0.5ml. of a solution of the test compound and 0.5ml. of a 0.2% (v/v) suspension of rabbit erythrocytes. All components were prepared in buffered saline, pH 8.5. The final concn. of compound (VIII) was 0.009mm (Δ), 0.020mm ($\square$), 0.034mm ($\bullet$), 0.036mm (Δ), 0.063mm ($\blacksquare$) or 0.103mm ($\bullet$). The reaction was started by the addition of the erythrocyte suspension immediately after the inhibitor and the α -toxin had been mixed. As a control, α -toxin was tested in the absence of compound (VIII) ($\circ$).

Fig. 2. Effect of concentration of compound (VIII) on maximum rate of haemolysis ($R_{\max}$), time to 50% haemolysis (t_1) and lag period (τ). The experimental procedure was as described for Fig. 1. The values of $R_{\max}$ (●), t_1 (Δ) and τ (□) were determined from the sigmoid haemolysis curves as described in the Materials and Methods section.

inhibitor is shown for compound (VIII) in Fig. 2. Similar curves were drawn from the results for all the compounds tested. From these, values for the concentration causing a 50% decrease in $R_{\max}$, a doubling of τ and a doubling of t_1 , compared with the control runs in absence of inhibitor, could be determined. These values are shown for all the compounds tested at pH 8.6 in Table 1; most of the compounds in groups 1 and 2 were also tested at pH 6.8, and these results are shown in Table 2.

At pH 8.6, group 2 contained the most potent inhibitors of haemolysis. Compounds (VII) and (VIII) were particularly active, causing a 50% decrease in $R_{\max}$ at concentrations of 0.023 mM and 0.014 mM respectively. These compounds were about ten times as inhibitory as the most active inhibitors in group 1 at this pH value. The compounds in group 3 were all poorly inhibitory, concentrations in excess of 8 mM being necessary to cause a 50% decrease in $R_{\max}$. Compound (III) in group 1 and compounds (X), (XI) and (XII) in group 2 were considerably less inhibitory than the other compounds in these groups. The significance of these results in relation to the chemical structures of the compounds is discussed below. The 50% decrease in $R_{\max}$ was the most sensitive indicator of inhibitory activity; in all cases higher concentrations were required to cause a doubling of τ and t_1 than to cause a 50% decrease in $R_{\max}$.

Table 1. *Inhibition of haemolytic activity of crude staphylococcal α -toxin by aromatic polysulphonic acids at pH 8.6*

The data were obtained from plots of $R_{\max}$, τ and t_1 against concentration of the test compound, such as those shown for compound (VIII) in Fig. 2.

Compound	Concn. (mm) causing 50% decrease in $R_{\max}$.	Concn. (mm) at which lag time (τ) was doubled	Concn. (mm) at which time to 50% haemolysis (t_1) was doubled
Group 1			
(I)	0.80	1.2	1.1
(II)	0.14	0.20	0.40
(III)	8.5	12.0	13.0
(IV)	0.16	—	8.6
(V)	Could not be tested because it was haemolytic at pH 8.6		
Group 2			
(VI)	0.48	—	—
(VII)	0.023	0.035	0.035
(VIII)	0.014	0.015	0.017
(IX)	0.80	0.70	0.50
(X)	2.0	4.4	3.8
(XI)	1.5	2.8	2.6
(XII)	4.8	5.0	7.0
Group 3			
(XIII)	15.0	> 20.0	> 20.0
(XIV)	11.0	18.0	> 20.0
(XV)	8.4	> 20.0	> 20.0
(XVI)	> 20.0	> 20.0	> 20.0
(XVII)	> 20.0	> 20.0	> 20.0

Table 2. *Inhibition of the haemolytic activity of crude staphylococcal α -toxin by aromatic polysulphonic acids at pH 6.8*

The data were obtained in the same way as described in Table 1.

Compound	Concn. (mm) causing 50% decrease in $R_{\max}$.	Concn. (mm) at which lag time (τ) was doubled	Concn. (mm) at which time to 50% haemolysis (t_1) was doubled
Group 1			
(I)	0.70	2.6	1.5
(II)	0.10	0.20	0.20
(IV)	0.030	0.040	0.060
(V)	0.013	0.017	0.012
Group 2			
(VI)	Could not be tested because it was haemolytic at pH 6.8		
(VII)	0.096	0.40	0.30
(VIII)	0.030	0.23	0.19
(IX)	0.35	0.60	0.60
(X)	5.8	7.0	11.0
(XI)	4.1	9.0	5.8
(XII)	6.0	10.0	8.0

Table 3. *Effect of premixing compound (I) with crude staphylococcal α -toxin before the addition of rabbit erythrocytes*

The reaction mixture was as described in the Materials and Methods section. The iso-osmotic phosphate-buffered saline, α -toxin dilution (20 M.H.D.) and solution of compound (I) were mixed for various times up to 60 min. before the addition of the rabbit erythrocyte suspension. The pH was 8.6 and the temperature of incubation was 25°.

Concn. of compound (I) (mm)	Time of premixing (min.)	$R_{\max}$ (min. ⁻¹)
0.07	0*	0.461
	60	0.320
1.399	0*	0.202
	15	0.159
	30	0.141
	60	0.103
3.498	0*	0.101
	15	0.053
	30	0.040
	60	0.021

* 0 min. represents the minimum possible time of premixing before the addition of rabbit erythrocytes.

At pH 6.8 compounds (I) (suramin) and (II) were almost as inhibitory as at pH 8.6, but compound (IV) was considerably more inhibitory at the lower pH. Moreover, compound (V), which could not be tested at pH 8.6 because it was haemolytic at that pH, proved to be the most inhibitory of all the compounds at pH 6.8; a concentration of 0.013 mm gave a 50% decrease in $R_{\max}$. In group 2, compounds (VII) and (VIII) were less inhibitory than at pH 8.6, but compound (IX) was more inhibitory at pH 6.8 than at pH 8.6. Compounds of group 3 were not tested at pH 6.8.

Although the inhibitory activity of most of the compounds was influenced by pH, pH-dependence was not as striking as that reported for the effect of suramin on certain enzymes (Wills & Wormall, 1950); for these enzymes inhibition was only observed at acid pH and a change of 0.4 pH unit resulted in an increase in inhibition from 0 to 100%.

The effect of premixing α -toxin (20 M.H.D.) with three different concentrations of compound (I) for periods of up to 1 hr. before the addition of the erythrocyte suspension to the reaction mixture is shown in Table 3. At all three concentrations

increased time of premixing caused an increase in the degree of inhibition, as can be seen from the decrease in $R_{\max}$.

DISCUSSION

Haemolysis of rabbit erythrocytes by staphylococcal α -toxin presents a rapid and fairly specific method of assessing the effect of possible inhibitors. The current view of the mechanism of lysis by α -toxin (Marucci, 1963; Cooper *et al.* 1964a; Madoff, Cooper & Weinstein, 1964) suggests that the α -toxin interacts with a component of the cell membrane during the prelytic lag period, resulting in an alteration of the permeability characteristics of the membrane and subsequent lysis of the cell. Since aromatic polysulphonic acids caused an increase in τ , in addition to a decrease in the rate of haemolysis ($R_{\max}$), it seems likely that these compounds act by preventing the interaction of α -toxin with its substrate or receptor; the relatively low concentrations required for inhibition by the most effective inhibitors and the influence of small differences in chemical structure of the test compounds indicate that inhibition was not due to osmotic stabilization of toxin-damaged erythrocytes. Moreover, the increased inhibition observed when α -toxin was premixed with compound (I) for increasing time-intervals before the addition of erythrocytes points to an action of the inhibitor on the toxin rather than on erythrocytes. This was supported by the finding that inhibition of the lethal effect of the toxin was also time-dependent (Arbuthnott *et al.* 1968). Some degree of interaction between the test compounds and erythrocytes, however, cannot be totally excluded, even though results for premixing of compound (I) with erythrocytes showed no significant increased inhibition relative to results where compound (I) and erythrocytes were added (effectively) simultaneously to the reactant system.

Previous extensive studies have shown that suramin (compound I) binds to plasma proteins and inhibits certain enzymes, probably by interaction of the sulphonic acid groups with the basic groups of the protein (Spinks, 1948; Wilson & Wormald, 1949; Town, Wills, Wilson & Wormald, 1950; Wills & Wormald, 1950). Such a mechanism may well explain the inhibition of α -toxin, especially by compounds of group 1, although the results of the present study suggest that the sulphonic acid groups alone do not govern the degree of inhibition. Since compound (III), which can be considered as 'half suramin', is at least ten times less inhibitory than the other compounds of group I, it appears that both the aromatic polysulphonic acid chains are necessary for maximum inhibitory activity in this group.

Wills & Wormald (1950) showed that the enzymes inhibited by suramin and related compounds fell into two groups. Those belonging to group A were inhibited only at acid pH and the inhibition was very sensitive to small changes in pH; in group B inhibition was also observed at alkaline pH and was much less sensitive to changes in pH. In the present study compounds (I) and (II) were almost equally inhibitory at both pH 6.8 and 8.6, and although compound (IV) was more inhibitory at pH 6.8 than at pH 8.6 it was still an efficient inhibitor at pH 8.6. On the basis of these limited pH studies it would seem that inhibition of staphylococcal α -toxin resembles that of group B in the study of Wills & Wormald (1950).

Within group 2 the presence of the dichlorotriazinyl ring and the benzenediazonium ring played an important part in inhibition. For instance, at pH 8.6 compound (VI) was considerably less inhibitory than compound (VIII), from which it differs only in the position of the dichlorotriazinyl ring. Also, compound (IX), which has no triazinyl ring, was considerably less inhibitory than compounds (VII) and (VIII). Thus the presence of the dichlorotriazinyl ring and its position in the molecule markedly affects inhibitory activity in group 2.

The presence of a sulphonic acid group on the benzenediazonium ring did not affect inhibitory activity at pH 8.6; both compound (VII) and compound (VIII) were almost equally inhibitory at this pH. At pH 6.8, however, the presence of a sulphonic acid group in this position did confer greater inhibitory activity. Removal of the benzenediazonium ring [compounds (X), (XI) and (XII)] resulted in a considerable loss of inhibitory activity, indicating that the presence of this group was essential for good inhibitory activity in group 2.

The compounds of group 3 represent simpler chemical structures that do not contain the naphthalene ring system. These were all poor inhibitors, indicating that the presence of sulphonic acid groups or of a triazinyl ring does not alone confer inhibitory activity.

The present findings have made possible the selection of fairly potent inhibitors of staphylococcal α -toxin that have proved useful in studying the inhibition of the lethal action of the α -toxin *in vivo* (Arbuthnott *et al.* 1968). Moreover, knowledge of the nature of the interaction between staphylococcal α -toxin and inhibitory aromatic sulphonic acids may lead to some understanding of the active site of the toxin.

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