Effect of thiol compounds on human complement component C4

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Thiol compounds have been investigated as inhibitors of the covalent binding reaction of human complement protein C4 using Sepharose–C1s as a combined activating and binding surface. *o*- and *p*-substituted aminothiophenols are equally effective inhibitors, whereas the *m*-substituted compound is a less potent inhibitor. The anti-hypertensive drug captopril is

also shown to inhibit the covalent binding reaction. A comparison of the effects of these compounds on the covalent binding reaction of isolated C4A and C4B has been made. Results suggest that a Pro-to-Leu substitution in C4B is likely to account for the differences in inhibitory potency of C4B compared with C4A observed with the aromatic inhibitors.

INTRODUCTION

The complement system is closely involved in the clearance of immune complexes [1]. When the complement cascade is activated through the classical pathway, components C1, C2 and C4 become activated and the complex enzyme C3 convertase, consisting of C4b2a, is generated on the complement-activating surface. The C3 that is activated becomes bound in turn to the immune complex surface. Covalent binding of C3 and C4 to the surface of immune complexes occurs through reaction of the internal thiolester in C3 and C4 with amino or hydroxy groups on the immune complex [2]. The role of complement activation in clearance of immune complexes appears to be physiologically important, as individuals who lack the early complement components are at increased risk of systemic lupus erythematosus [3]. In this autoimmune disease, immune complexes become deposited at inappropriate tissue sites, including small blood vessels and kidney glomeruli, with resulting tissue damage.

Complement component C4 is encoded at two polymorphic loci, C4A and C4B, within the class III region of the major histocompatibility complex. The products of the two genes are very similar and the isotypic region has been located to four amino acids within a group of six out of a total of 1722 in the complete C4 sequence [4]. The C4A and C4B gene products differ in the reactivity of the thiol ester site on activation. The C4A gene product has been shown to be more reactive than the C4B gene product with amino nucleophilic groups [5,6]. The physiological roles of C4A and C4B may also differ, as it has been observed that systemic lupus erythematosus is associated with non-functioning alleles at the C4A locus rather than at the C4B locus [7], and the C4A protein has been demonstrated to restore an immune response to C4-deficient guinea pigs which are immunologically compromised [8]. Guinea pig C4 has been shown in previous studies to have an activity profile like that of human C4A [9].

Several drugs are associated with toxic side-effects resembling systemic lupus erythematosus [10]. These include the anti-hypertensive drug hydralazine and the anti-rheumatic drug penicillamine [11]. It has been demonstrated that both drugs interfere with the covalent binding reaction of C4 [2]. Both hydralazine [12] and penicillamine [13] are more inhibitory with C4A than with C4B. The inhibition of the covalent binding of

C4A by penicillamine occurs over the therapeutic concentration range, and it has previously been observed that penicillamine causes a decrease in the deposition of C3 in arthritic joints [14]. On the basis of these results it has been proposed that inhibition of C4 covalent binding by penicillamine contributes to both therapy and toxicity with the drug [13].

Penicillamine is an α -amino β -thiol compound [15]. In order to ascertain which aspects of penicillamine are important in promoting interaction of the drug with complement component C4, a range of chemically similar compounds has been investigated. These compounds show that it is not necessary for the amino and thiol groups to be on adjacent carbon atoms, as they are in penicillamine, to generate potent inhibition of C4. The importance of the thiol group in inhibition of the covalent binding reaction of C4 is demonstrated. Captopril, an angiotensin-converting enzyme inhibitor which is used as an anti-hypertensive agent, is another thiol drug with immunotoxic side effects [16]. Here it is demonstrated also to be an inhibitor of the covalent binding reaction of C4.

MATERIALS AND METHODS

Coupling of protein to Sepharose

C1s was isolated from human serum and coupled to CNBr-Sepharose (Pharmacia, Milton Keynes, U.K.) as previously described [17] to give a concentration of 3 μ g of C1s per ml of Sepharose. Mouse monoclonal antibodies against C4c (LOO1) [18] and against C4d (LOO3) [19] were obtained as ascites fluid from the Commonwealth Serum Laboratories, Parkville, NSW, Australia. Antibodies were partially purified by ammonium sulphate precipitation [20], resuspended at a concentration of 15 mg/ml and then dialysed into 500 mM NaCl and 20 mM sodium phosphate, pH 7.5, for coupling to CNBr-Sepharose. The mixture of CNBr-Sepharose (1 ml packed volume per 10 mg of protein) and antibodies was shaken at 20 °C for 1 h, which resulted in more than 90% of the protein binding to the resin. Longer coupling reactions at pH 8.5 were found to decrease the binding capacity of the antibody affinity columns for C4. The derivatized Sepharose was used for affinity columns (2–5 ml) after saturating the remaining binding sites on CNBr-Sepharose with 1 M Tris/HCl, pH 7.5 (16 h, 4 °C).

Figure 1 Inhibition of the covalent binding of human C4 to Sepharose-C1s by aminothiophenols

o- (▲), *m*- (●) and *p*- (○) aminothiophenols were tested as inhibitors of covalent binding of ^{125}I -labelled C4 as described in the text. The percentage inhibition was determined by comparison with the inhibition of binding in the presence of 20 mM hydroxylamine, which was taken to be 100%.

Purification of human C4

Human C4 was purified by a two-step procedure using affinity chromatography followed by ion exchange on a Mono-Q HR5/5 column (Pharmacia). All chromatography was performed at 4 °C. C4A and C4B were purified together on an LOO1 monoclonal antibody column, and were separated using an LOO3 monoclonal antibody column. Affinity columns were prepared and eluted essentially as described previously [19]. Pooled human plasma (2–3 ml, with approximately equal amounts of C4A and C4B) containing 5 mM EDTA was made 1 mM with respect to phenylmethanesulphonyl fluoride (PMSF) and centrifuged (13000 rev./min, 5 min) in a Fisons Microfuge at 4 °C. The plasma was loaded on to the appropriate column, pre-equilibrated with 50 mM Tris, 50 mM sodium phosphate, 12.5 mM sodium tetraborate, 2.5 mM EDTA, 0.1 mM PMSF and 0.01 % sodium azide at pH 7.0. After 30 min at 4 °C, the column was washed with 100 mM Tris, 100 mM sodium phosphate, 25 mM sodium tetraborate, 5 mM EDTA, 0.2 mM PMSF and 0.02 % sodium azide at pH 8.5 until the absorbance of the eluate at 280 nm was zero. C4 was eluted with a linear pH gradient made from 15 ml of the pH 8.5 buffer and 15 ml of the same buffer adjusted to pH 11.5, finishing with a pH 11.5 buffer wash. C4A is eluted from the LOO3 column at pH 9.0–9.5, whereas C4B eluted between pH 9.5 and 11. The appropriate C4 pools were then dialysed into 20 mM Tris, 5 mM EDTA, 0.02 % sodium azide and 0.2 mM PMSF at pH 7.5. The samples were further purified by f.p.l.c. using ion exchange on a Mono-Q column. The column was initially washed with 20 mM Tris, 5 mM EDTA, 0.02 % sodium azide, 0.2 mM PMSF, 50 mM sodium ϵ -amino hexanoate and 1 M NaCl at pH 7.5 (buffer B), and then with the same buffer without NaCl (buffer A). The sample was applied in 2 ml aliquots at 1 ml/min. The column was washed with 10 % (v/v) buffer B until the absorbance at 280 nm was zero. A linear gradient to 60 % (v/v) buffer B was then applied in 30 ml, followed by a wash (5 ml) at 100 % (v/v)

buffer B to complete the elution. Fractions were monitored by SDS/PAGE, and C4 concentrations were estimated from absorbance at 280 nm.

Binding of C4 to Sepharose-C1s

Pure C4, C4A and C4B (10–30 μg) were radiolabelled with Na^{125}I (500–700 μCi) with one Iodobead (Pierce) as catalyst in a total volume of 100 μl of 20 mM sodium phosphate, pH 7.5, containing 140 mM NaCl, as previously described [17]. The specific radioactivity of C4 samples was 0.2–0.6 $\mu\text{Ci}/\mu\text{g}$ of C4.

Covalent binding of ^{125}I -C4b, ^{125}I -C4Ab or ^{125}I -C4Bb to Sepharose-C1s [100 μl of a 50 % (v/v) slurry] was determined in triplicate [17] in a total volume of 150 μl of 20 mM sodium phosphate and 140 mM NaCl. Dimethyl sulphoxide (DMSO) (1 %, v/v) was used as solvent for the aminothiophenol test compounds and was included in all control samples. After 30 min at 37 °C, the reaction was stopped by addition of 200 μl of 200 μM Tris/HCl, pH 6.8, containing 2 % (w/v) SDS and 8 M urea. Sepharose 4B (Pharmacia) [200 μl of a 50 % (v/v) slurry] in 20 mM sodium phosphate and 140 mM NaCl was then added to increase the bulk volume of Sepharose and to minimize losses during the washing steps. Centrifugation and washing steps were performed as described previously [17]. The final pellet was resuspended in 100 mM NaCl (200 μl) and was then transferred to a clean tube for measurement of bound radioactivity. Complete inhibition of ^{125}I -C4b binding was determined by including 20 mM hydroxylamine, pH 7.5, in the initial incubation mixture with Sepharose-C1s.

SDS/PAGE analysis

To distinguish between C4A and C4B, polyacrylamide gels were prepared with a ratio of acrylamide/bisacrylamide of 1:0.006 [21], and were analysed by either autoradiography or silver staining [22].

Thiol compounds

Aminothiophenol compounds were stored at 20 °C in a desiccator containing a loosely capped vial of 100 mM dithiothreitol, to maintain a reducing environment.

The aromatic compounds were initially dissolved as a stock solution in DMSO and were then diluted 100-fold in 20 mM sodium phosphate and 140 mM NaCl, pH 7.5. The stock solutions and dilutions were made fresh each day. Stock solutions of penicillamine and captopril were made fresh each day in 20 mM sodium phosphate and 140 mM NaCl, pH 7.5.

Measurement of reduction of C4

Pure ^{125}I -C4 (specific radioactivity 0.4 $\mu\text{Ci}/\mu\text{g}$ of C4) in 20 mM sodium phosphate, pH 7.5, containing 140 mM NaCl was incubated (in 30 μl) with the test compound for 30 min at 37 °C. Iodoacetamide was then added to a final concentration of 10 mM in the same buffer and the mixture was incubated for a further 10 min at 37 °C. An equal volume of 200 mM Tris/HCl, pH 6.8, containing 2 % (w/v) SDS and 8 M urea was then added and samples were incubated at 37 °C for 20 min and then analysed by SDS/PAGE and autoradiography.

Titration of aminothiophenols

The pH of 20 ml of 1 mM aminothiophenol in DMSO was determined. The pH of the solution was then monitored on

Table 1 Inhibition of binding of ^{125}I -C4 to Sepharose-C1s

Nucleophilicities were calculated as in the Materials and methods section. n.d. not determined.

Structure	Name	IC ₅₀ for C4b binding	Nucleophilicity (mM ⁻¹ · min ⁻¹)
	Penicillamine	220 μM	n.d.
	<i>o</i> -Aminothiophenol	90 μM	418.3
	<i>m</i> -Aminothiophenol	510 μM	1120.0
	<i>p</i> -Aminothiophenol	88 μM	5484.4
	<i>o</i> -Aminophenol	1.15 mM	n.d.
	<i>o</i> -Aminobenzoic acid	37% at 5 mM	n.d.
	Captopril	1 mM	n.d.

addition of 10 μl aliquots of either 10 mM NaOH or 10 mM HCl. The pK_a was determined graphically from the plot of pH against the volume of counterion added.

Determination of nucleophilicity of aminothiophenols

The nucleophilicity of aminothiophenols was ascertained by monitoring the rate of formation of *p*-nitrophenol from *p*-nitrophenol acetate. The substrate, *p*-nitrophenol acetate (15 mM), was made up fresh in DMSO and kept at 4 °C. In the final reaction mixture, 30 μl of the aminothiophenol, from a stock solution in DMSO, was made up to 2.9 ml with 14.3 mM sodium phosphate, pH 7.5; in 27.9% DMSO, to which 100 μl of the substrate solution was added. The rate of formation of *p*-nitrophenol was calculated as the rate of change in absorbance at

400 nm. An accurate concentration of the nucleophilic species at pH 7.5 was determined using the Henderson–Hasselbach equation and the previously determined pK_a values. The nucleophilicity can be calculated from the equation:

$$\text{Nucleophilicity} = \frac{\text{Rate of formation of } p\text{-nitrophenol}}{\text{Concentration of nucleophile}}$$

RESULTS

Effects of thiol compounds on C4 and on C1s cleavage of C4

No difference was observed between the extent of C4 cleavage by C1s in the presence or absence of any of the compounds tested, as measured by SDS/PAGE of reduced and alkylated C4 and C1s-cleaved C4.

Table 2 Comparison of inhibition of covalent binding of ^{125}I -C4Ab and ^{125}I -C4Bb to Sepharose-C1s

Inhibitor	IC ₅₀ for binding to Sepharose-C1s (μM)		Ratio C4B/C4A
	C4A	C4B	
<i>o</i> -Aminothiophenol	70	155	2.2
Penicillamine	180	1500	8.3

Reduction of interchain disulphide bridges was assessed, by SDS/PAGE of samples prepared for electrophoresis with alkylation only, for all of the thiol compounds used. When ^{125}I -C4 was incubated with *o*-aminothiophenol, no reduction of interchain disulphide bridges was observed up to $350\ \mu\text{M}$. At $500\ \mu\text{M}$, approx. 30 % of the C4 migrated as reduced C4 (i.e. as α , β and γ chains) while the rest of the C4 was unchanged and migrated as a 205 kDa band. The extent of reduction was measured from counting the radioactivity associated with the α , β and γ chains.

Captopril and penicillamine up to 5 mM caused no reduction to interchain disulphide bridges in C4.

Effect of thiol compounds on the covalent binding of C4 to Sepharose-C1s

The binding of ^{125}I -labelled C4 to Sepharose-C1s as a combined activating and binding surface was inhibited in a dose-dependent manner by *o*-aminothiophenol, *p*-aminothiophenol and *m*-aminothiophenol (Figure 1). The inhibition by the *o*- and *p*-substituted compounds was indistinguishable, while the *m*-substituted compound was a less potent inhibitor, with 50 % inhibition of binding occurring at $510\ \mu\text{M}$. The hydroxyl compound *o*-aminophenol is a less potent inhibitor of the covalent binding reaction of C4 and *o*-aminobenzoic acid is a very poor inhibitor (Table 1).

The IC₅₀ for inhibition of pooled C4 binding to Sepharose-C1s by penicillamine was $220\ \mu\text{M}$, while the effect of captopril was less potent, with 50 % inhibition of binding occurring at 1 mM.

Effect of thiol compounds on C4A and C4B

Penicillamine has been shown previously to be a more potent inhibitor of the covalent binding of C4A than of C4B to a complement-activating surface consisting of erythrocytes as antigen [17]. When the same experiment was carried out with Sepharose-C1s as the C4-activating surface, the same preference for inhibition of C4A was observed (Table 2). With penicillamine as inhibitor the inhibition of C4A covalent binding was 8-fold greater than the inhibition of C4B binding (Table 2), but the difference between inhibition of C4A and C4B binding was only 2-fold with *o*-aminothiophenol as inhibitor.

DISCUSSION

The results presented show that inhibition of the covalent binding reaction of C4 by arylamine compounds is greatly increased by the presence of a thiol group, as demonstrated when the inhibitory potencies of aminophenol and aminothiophenol are compared (Table 1). It has been observed previously that penicillamine is a potent inhibitor of the covalent binding reaction of C4 to a surface consisting of C1s bound to an antibody-antigen complex

with erythrocytes as antigen [13]. Penicillamine is also shown here to be a potent inhibitor of the covalent binding of C4 to Sepharose-C1s as the combined activating and binding surface.

It has been suggested that inhibition of the covalent binding reaction of C4 by penicillamine, an α -amino β -thiol compound, results in formation of a thiazoline ring complex between C4 and penicillamine [13]. Both *o*- and *p*-aminothiophenol are equally potent inhibitors, and while *o*-aminothiophenol could form a thiazoline ring, it is most unlikely that *p*-aminothiophenol could form such a compound. It is likely to be the nucleophilicity of the thiol group of *p*-aminothiophenol that is important in promoting interaction with the thiol ester in C4 (Table 1). An increasing inhibitory potency of inhibitors of the covalent binding reaction of C3 [23] and C4 [24] has already been noted to correlate with their nucleophilicity. The relative potencies of the *p*- and *m*-aminothiophenols as inhibitors of the covalent binding reaction of C4, compared with their relative nucleophilicities (Table 1), substantiates this suggestion. However, the potency of *o*-aminothiophenol is likely to be due to a combination of the nucleophilicity of this aromatic α -amino β -thiol and the possibility of the formation of a thiazoline ring with the carbonyl group of the thiolester of C4.

The effect of thiol compounds on the covalent binding of the different isotypes of C4 shows that the difference in inhibition of C4A and C4B binding observed with arylaminothiols compounds is much less than the difference observed with the less hydrophobic aminothiols such as penicillamine and cysteine [13]. With penicillamine and cysteine, C4A is inhibited 8–9 times more effectively than is C4B; however, with the arylaminothiophenols, the inhibitory potency towards C4A is only 2-fold greater than the inhibitory potency towards C4B. It has been observed that the difference between C4A and C4B which contributes to the binding specificity difference between the two isotypes resides in a sequence of six amino acids, which is Pro-Cys-Pro-Val-Leu-Asp in C4A but Leu-Ser-Pro-Val-Ile-His in C4B[4]. It has been observed by site-directed mutagenesis and by comparison of the active sites of C4 and its homologues from different species that the aspartate residue in the specificity-conferring region promotes the increased interaction of C4A with amino nucleophilic groups [25]. It has been proposed that the cysteine/serine interchange promotes the interaction of C4A with thiol groups [13]. However, it would seem that, for more hydrophobic aminothiol compounds, the presence of a thiol group in the specificity-determining region of C4A is compensated for by the substitution of a proline in C4A for the more hydrophobic leucine in C4B.

It has been suggested that the effect of penicillamine in inhibiting the covalent binding reaction of C4 could be important for the therapeutic effect of penicillamine as an anti-arthritis drug [13]. It has also been suggested that drug-induced immunotoxic side effects, which result in immune complex deposition at inappropriate sites, could be due to the inhibitory effect of these drugs on the covalent binding reaction of C4 [2]. These drugs include penicillamine and hydralazine. It has been demonstrated that captopril also inhibits the covalent binding reaction of C4 (Table 1). Captopril (Table 1) and hydralazine [17] inhibit the covalent binding of C4 within the same concentration range. They are much poorer inhibitors of the covalent binding reaction of C4 than is penicillamine. This mirrors the incidence with which toxicity related to immune complex handling occurs with these different drugs. It has been suggested that up to 12 % of patients experience adverse effects with hydralazine [26], while captopril causes adverse side-effects relating to immune complex handling in less than 10 % of users. However, penicillamine causes serious proteinuria in up to 30 % of individuals taking the drug [15]. The propensity of these drugs to

induce immune complex handling problems may be related to their potency as inhibitors of C4 covalent binding.

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