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COMPLEMENT INHIBITION BY AMIDINES AND GUANIDINES—*IN VIVO* AND *IN VITRO* RESULTS

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Abstract—Amidines and guanidines have been reported to be effective inhibitors *in vitro* of complement. This study reports the *in vivo* activity of four such compounds, 4-aminobenzamidine, 3,4-dimethoxybenzamidine, 4-methyl-2-guanidinoquinazoline and pentamidine, as reflected in their ability to inhibit the Forssman reaction, a model of complement-mediated tissue damage. Inhibitory concentrations of each compound were first determined using whole guinea pig serum. In contrast to two other, known complement inhibitors, cobra venom factor and fumaropimaric acid, these compounds failed to block the Forssman reaction after intravenous administration at greater than their computed inhibitory concentrations. In addition, the serum from amidine-dosed guinea pigs failed to show complement inhibition, indicating that inhibitory concentrations had not been achieved *in vivo*. Confirming this, 4-aminobenzamidine was shown to partition out of blood into an apparent volume of distribution of *ca.* 2.7 liters/kg less than 2 min after an intravenous dose. Other amidines and guanidines, including pentamidine, show a correspondingly large volume of distribution. We conclude that this class of inhibitors must undergo structural modifications designed to produce adequate and sustained plasma concentrations before *in vivo* anticomplementary activity can be expected.

The complement system is intimately involved not only in host defense against infectious agents, but also in the pathogenesis of immune-complex diseases such as acute glomerulonephritis [1], rheumatoid arthritis [2] and lupus erythematosus [1]. In these diseases, agents which inhibit the complement system would be expected to ameliorate the acute pathological changes induced by immune complexes. Indeed, preliminary evidence suggests that at least one complement inhibitor, tranexamic acid, may be beneficial in systemic lupus erythematosus [3].

Among the *in vitro* inhibitors of complement that might possess clinical utility, the amidines and guanidines appear particularly attractive. They are low molecular weight compounds and, therefore, not intrinsically antigenic. The relationships between their chemical structures and complement inhibitory properties *in vitro* have been developed extensively [4–6]. The sites of action of the amidines on the complement cascade have been clarified [7–11]. Despite the apparent potency of these agents as *in vitro* complement inhibitors, there are no reports of their effects on the complement system in the intact animal. It was, therefore, the objective of this study to determine whether the inhibition reported for this class of compounds could be translated into *in vivo* anticomplement activity.

The studies of Bing [7] have demonstrated that the amidines are effective inhibitors of the first component of complement (Cls) although they are known to inhibit several other steps in the complement pathway [8, 10, 11]. His results suggest that the amidines should be tested *in vivo* in an immune model dependent upon the classical C1, 4, 2 pathway. There are two well-known models for complement-dependent tissue damage mediated by immune complexes, the Arthus and the Forssman reactions. The work of May and Frank [12] demonstrated that only the Forssman reaction appeared absolutely dependent on the classical pathway; i.e. in C4-deficient guinea pigs there exists a complete block of Forssman reactivity; normal reactivity is restored by infusion of purified C4. Consequently, the Forssman reaction was chosen as the system in which to test the ability of the amidines and guanidines to inhibit antibody-dependent, complement-mediated tissue damage.

METHODS

Complement inhibitors. Cobra venom factor was purchased from Cordis Laboratories, Miami, FL, and 4-aminobenzamidine, from Sigma Chemical Corp., St. Louis, MO. 3,5-Dimethoxybenzamidine and fumaropimaric acid were prepared by Dr. George Milne of these laboratories. Pentamidine was a gift from May & Baker, Dagenham, Essex, England. 4-Methyl-2-guanidinoquinazoline was purchased from Aldrich Chemical Corp., Madison, WI.

Assay of complement activity. Complement activity was determined by a modification of the original kinetic assay of Plescia *et al.* [13]. One ml of sheep red blood cells (SRBC), 1×10^9/ml, was mixed with 0.2 ml of a 1/200 dilution of hemolysin and 0.8 ml of Dulbecco's phosphate-buffered saline, DPBS (Microbiological Associates, Bethesda, MD), incubated 0.5 hr at 37°, washed twice with DPBS, and resuspended in 2 ml DPBS containing 1×10^{-3} M Mg^{2+} and 1.5×10^{-4} M Ca^{2+}. Guinea pig serum from an appropriate test or control animal was mixed with an equal volume of the sensitized SRBC at 4° and transferred to a 37° bath. At 4-min intervals, 0.1-ml aliquots were

removed and quenched in 3 ml of ice-cold DPBS containing 0.01 M EDTA. The unlysed SRBC were removed by centrifugation and the absorbance of the supernatant fraction was read at 541 nm. Inhibition was expressed numerically as the per cent decrease in absorbance at 541 nm of test vs control serum at the 20-min time point.

Assay of inhibitor activity in vivo. The complement inhibitors were dissolved in physiological saline and the solutions adjusted to pH 7. Guinea pigs were dosed intravenously with the inhibitor. At predetermined time intervals, the guinea pigs were bled by cardiac puncture. The blood was allowed to clot, and serum complement was determined by the assay described above.

Assay of inhibitor activity in vitro. Inhibitor activity was assayed *in vitro* by modifying the addition of indicator SRBC. One ml of sensitized SRBC (1×10^9/ml) was added to 1 ml of a predetermined concentration of inhibitor at pH 7 in DPBS with Ca^{2+} and Mg^{2+}. The inhibitor activity was then determined by adding 1 vol. of the normal guinea pig serum to 1 vol. of this mixture and assaying the complement activity as described above.

Systemic Forssman reactions. Anti-Forssman antibody was prepared by the method of Taliaferro and Taliaferro [14] using splenectomized rabbits. The minimum lethal dose (LD_{100}) of anti-Forssman antibody was determined by intravenous injection of increasing amounts of antibody into guinea pigs until the lowest dose that would kill three of three animals was reached. The LD_{100} was determined for each antibody preparation and routinely resulted in death at 6–8 min post injection. The effect of an inhibitor was determined by intravenous injection of the inhibitor 5 min prior to the administration of the LD_{100} concentration of anti-Forssman antibody. Survival was scored at 15 min.

Plasma levels of 4-aminobenzamidine. The drug was administered i.v. to guinea pigs, as a solution in physiological saline, at a dose level of 125 mg/kg, and serial bleedings (*ca.* 1 ml) were performed[15] at 3, 5 and 9 min post dose. Control blood was obtained similarly from normal guinea pigs. The drug was extracted from serum, derivatized with hexafluoroacetylacetone, and assayed by gas–liquid chromatography on a Microtek model MT-220 instrument equipped with a ^{63}Ni electron capture detector.* A standard curve correlating drug concentration with detector response was generated by fortifying control serum with the drug and processing these standards in parallel with each set of samples.

RESULTS

Complement inhibitors in vitro: *effective dose for 50 per cent inhibition.* Fumaropimaric acid, reported by Glovsky *et al.* [16] to be an inhibitor of complement both *in vitro* and *in vivo*, was studied first as a standard, low molecular weight reference compound. In agreement with the data of Glovsky *et al.* [17], fumaropimaric acid is an effective inhibitor of complement, but only at 3 mM or higher concentration (Table 1).

The effects of each of the five compounds tested as *in vitro* inhibitors of complement are summarized in Table 1. Pentamidine was the most active; it required a concentration of 1.5 mM to effect 50 per cent inhibition of the complement system. 4-Aminobenzamidine, 3,4-dimethoxybenzamidine and 4-methyl-2-guanidinoquinazoline were much less active. At 10 mM, they afforded only 57, 42 and 62 per cent inhibition respectively.

Complement inhibitors in vivo: *effect on the systemic Forssman reaction.* Cobra venom factor (CoVF) was studied as a standard *in vivo* inhibitor of complement-dependent reactions [18]. Twenty-four hours after i.v. administration of 0, 1, 5, 10 and 20 units CoVF to guinea pigs, 1 LD_{100} of anti-Forssman antibody was given. No animal receiving 0, 1 or 5 units CoVF survived the Forssman reaction; those receiving 10 or 20 units CoVF were protected. To determine the degree of complement inhibition necessary for survival, guinea pigs were dosed i.v. with 10 and 20 units CoVF. Twenty-four hours later they were bled and the sera saved. The guinea pigs were then challenged with 1 LD_{100} of anti-Forssman antibody. Again, all guinea pigs survived 1 LD_{100} of anti-Forssman antibody after 10 and 20 units CoVF. The complement activity of the pooled sera from three animals was assayed. At 10 and 20 units CoVF, substantial inhibition was found: 63 and 85 per cent respectively. In all, four separate determinations of the level of complement inhibition resulting from 10 units CoVF were performed in this fashion; 64, 39, 34 and 63 per cent inhibition of complement was found. Thus, as measured in this assay 34 per cent is the minimum inhibition of complement required for survival after administration of 1 LD_{100} of anti-Forssman antibody.

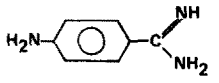
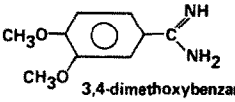
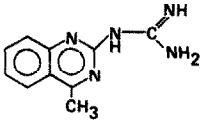
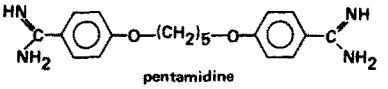
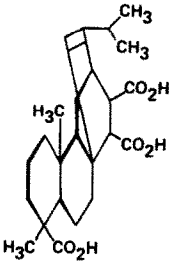
Fumaropimaric acid at 600 mg/kg i.v. was reported by Glovsky *et al.* [16] to allow guinea pigs to survive challenge with anti-Forssman antibody. The complement of a guinea pig dosed with 600 mg/kg i.v. of fumaropimaric acid and bled immediately was completely inhibited† and the guinea pig was able to survive 1 LD_{100} of anti-Forssman antibody when antibody challenge followed drug administration immediately.

The amidine and guanidine inhibitors were then tested for their ability to protect against lethal Forssman shock. Inhibitors were administered i.v., and, 5 min later, 1 LD_{100} of anti-Forssman antibody was given. Each inhibitor was tested at doubling doses up to the toxic limit. At 250 mg/kg, i.v., 4-aminobenzamidine was acutely lethal in guinea pigs; at 125 mg/kg, i.v., or less, it failed to protect against the anti-Forssman antibody (Table 2). Similar effects were found with 3,4-dimethoxybenzamidine, 4-methyl-2-guanidinoquinazoline, and pentamidine; at ≤50 per cent of the acutely lethal dose, the inhibitor afforded no protection against the Forssman reaction.

* Unpublished procedure of Dr. H. Fouda, Pfizer Central Research, Groton CT.

†This is the equivalent of 1.44 mmoles/kg. Assuming that the volume of distribution is the blood volume, *ca.* 0.1 liters/kg, then the nominal concentration would be 14 mM at zero time.

Table 1. *In vitro* complement inhibition

Structure	Conc. mM	Absorbance at 541 nm+ Control	Drug	% Inhib.‡
(I)  4-aminobenzamidine	0.1	.265	.254	4
	1.0	.265	.214	19
	2.0	.265	.181	31
	6.0	.265	.133	49
	10.0	.265	.112	57
(II)  3,4-dimethoxybenzamidine	3.0	.553	.480	13
	10.0	.553	.321	42
(III)  4-methyl-2-guanidinoquinazoline	2.0	.434	.404	7
	4.0	.434	.359	17
	6.0	.523	.450	14
	10.0	.434	.163	62
 pentamidine	0.5	.434	.410	5
	1.5	.600	.278	53
	6.0	.523	.058	100
(IV)				
(V)  fumaropimaric acid	1.0	.398	.320	19
	2.0	.398	.265	43
	3.0	.398	.105	72
	6.0	.398	.025	99

+Absorbance measured at 20 minutes.

‡Percent inhibition of SRBC lysis by inhibitor.

Table 2. Protection against lethan Forssman reaction by complement inhibitors

Complement Inhibitor	Dose mg/kg i.v.	Drug* Toxicity	Forssman** Survival
(I) 4-Aminobenzamidine	125	–	No
	250	+	
(II) 3,4-Dimethoxybenzamidine	150	–	No
	240	+	
(III) 4-Methyl-2-guanidinoquinazoline	200	–	No
	400	+	
(IV) Pentamidine	200	–	No
	400	+	
(V) Fumaropimaric acid	600	–	Yes
Cobra venom factor	10 units	–	Yes

*The dose at which inhibitor alone was toxic.

**The Forssman reaction was run 5 minutes after i.v. drug administration or 24 hours after cobra venom factor using 1 LD_{100} of anti-Forssman antibody. Survival of the Forssman challenge was scored at 15 minutes.

Effect of the amidines on complement activity in vivo. The inability of these compounds to protect against the Forssman reaction raised the question of whether (1) they failed to inhibit complement *in vivo* or (2) they inhibited complement *in vivo* yet failed to protect against the Forssman reaction for other reasons. Thus, the effect of 4-methyl-2-guanidinoquinazoline on complement *per se* was examined *in vivo*. Two guinea pigs were dosed at 200 mg/kg, i.v., and bled 5 min later. Two more were treated identically with 300 mg/kg and a third pair, pre-treated with 20 units CoVF, i.v., 24 hr earlier, served as positive controls. Control sera were obtained from two non-treated animals. The results are shown in Table 3. Whereas CoVF inhibited complement activity to the extent of 63 per cent, 4-methyl-2-guanidinoquinazoline at 200 and 300 mg/kg i.v. failed to depress complement activity. Similar results were obtained with 4-aminobenzamidine; at 125 mg/kg i.v., it failed to inhibit complement (Table 3). Since both agents inhibited complement *in vitro*, these results suggest that blood concentrations *in vivo* were not adequate for inhibition.

Serum levels of 4-aminobenzamidine. To test the hypothesis that the amidines fail to attain *in vivo* concentrations sufficient to inhibit complement, the serum levels of 4-aminobenzamidine were determined as a function of time. Three guinea pigs were injected intravenously with 4-aminobenzamidine, 125 mg/kg, and each animal was bled at appropriate times. Serum concentrations of 4-aminobenzamidine as a function of time are shown in Fig. 1. A linear regression analysis of the data from each animal permits calculation of a volume of distribution and of a rate constant for the disappearance of amidine from the serum. Derived pharmacokinetic parameters (Table 4) clearly indicate a very rapid distribution of intravenously administered drug out of the systemic circulation. The nominal mean serum concentration extrapolated to "time zero" (C_0) was 46.3 μg/ml (0.34 mM). From this, the apparent volume of distribution, V_d, was calculated to be 2.7 liters/kg, reflecting the virtually quantitative removal of drug from the circulating blood; less than 1.5 per cent of the total body burden of drug remained in the circulating plasma. Further, the disappearance of drug from the plasma must take place in a time period shorter than our earliest measurements, i.e. within seconds of intravenous administration.

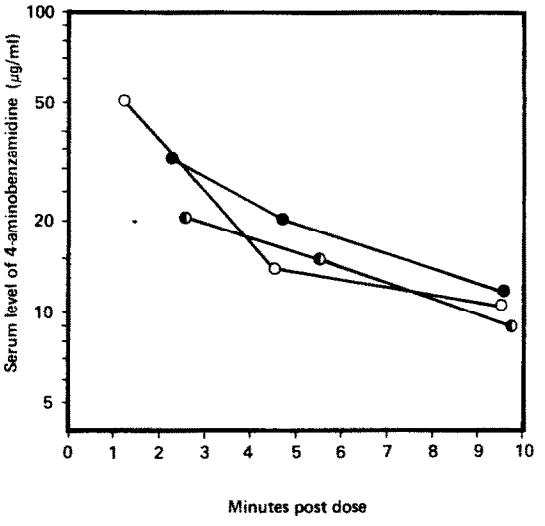


Fig. 1. Serum level of 4-aminobenzamidine as a function of time. Guinea pigs were dosed with 4-aminobenzamidine and bled at three successive time periods. The 4-aminobenzamidine serum concentrations were determined as described in Methods.

Table 4. Pharmacokinetic characteristics of 4-aminobenzamidine in the guinea pig

Animal number	Plasma C_0 (μg/ml)	Plasma half-life $t_{1/2}$(min)	Volume of distribution V_d(liters/kg^{-1})
01	68	2.9	1.84
02	45	4.8	2.78
03	26	6.7	4.81
Mean	46	4.3	2.70

Drug was administered at a dose of 125 mg/kg i.v.
C_0 = zero time intercept of plasma concentration.
V_d = Administered Dose/C_0.

Table 3. Complement inhibition after intravenous administration of inhibitor

Inhibitor	Dose	Complement Activity+		
		Absorbance at 541 nm		Percent‡ inhibition of SRBC lysis
		Control	Drug treated	
4-Methyl-2-guanidino-quinazoline (III)	200 mg/kg i.v.	.300	.279	7
	300 mg/kg i.v.	.275	.266	4
4-Aminobenzamidine (I)	125 mg/kg i.v.	.849	.759	10
Cobra venom factor	10 units i.v.	.328	.110	64

+ Control serum was taken from each guinea pig and immediately, inhibitor was administered intravenously; 5 minutes later each animal was bled again. The sera from one to three animals were pooled and complement activity was determined at a 1:1 final dilution of the sera.

‡Maximal percent inhibition achieved in a series of separate determinations.

DISCUSSION

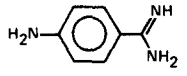
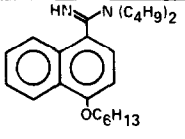
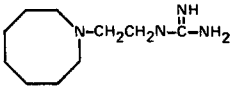
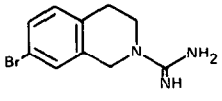
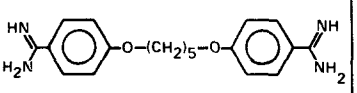
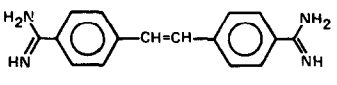
In these experiments we examined four inhibitors: 4-aminobenzamidine (I), reported by Asghar *et al.* [11] to be active against Cls at 1.35×10^{-3} M; 3,4-dimethoxybenzamidine (II) (active against Cls, our unpublished data); 4-methyl-2-guanidinoquinazoline (III), reported by Bing [7] to be active at 7.65×10^{-5} M; and pentamidine (IV), reported to be active by Asghar *et al.* [11] at 8.5×10^{-5} M. In our assay system, using a final complement dilution of 1:1 and a 20-min incubation period, we found 50 per cent inhibition at 6×10^{-3} M (I), 12×10^{-3} M (III) and 1.5×10^{-3} M (IV). All of the agents showed substantially less inhibition in our minimally diluted complement assay system than against isolated Cls. The diminished activity in our complement assay system may be attributable to the fact that the complement component which was inhibited, whether it was Cls or another component [8, 10, 11], is not rate limiting, and to the built-in amplification which occurs at several steps in the complement cascade.

Prior to examining the effects of the amidines on the Forssman reaction, CoVF was tested as a standard *in vivo* complement inhibitor. CoVF, at 10 units/kg of body weight, barely permitted guinea pigs to survive Forssman anaphylaxis. The animals experienced very obvious breathing difficulties and general weakness. At a higher dose, 20 units/kg, there were no observable effects from the injection of 1 LD_{100} of anti-Forssman antibody. Determination of the complement activity in the CoVF-treated guinea pigs showed that as little as 34 per cent inhibition of complement by 10 units/kg of CoVF could provide protection against lethal Forssman anaphylaxis. This suggested that, if the amidines could attain blood levels that would give 30–50 per cent complement inhibition, as measured by our *in vitro* assay, the guinea pig should have a good chance of surviving exposure to 1 LD_{100} of anti-Forssman antibody.

For amidines (I), (II), (III) and (IV), the required minimum plasma concentrations are computed to be 1.8×10^{-3}, 3.5×10^{-3}, 2.7×10^{-3} and 4.5×10^{-4} M respectively. Assuming a volume of distribution comparable to the blood volume (0.1 liter/kg), a minimum mg/kg dose for intravenous administration can be computed for each inhibitor. In every case, the inhibitors were tested intravenously at concentrations two to three times their computed inhibitory dose; nevertheless, each of the inhibitors failed to block the Forssman reaction (Table 2).* Further, when examined *in vitro*, sera obtained from guinea pigs dosed intravenously with two compounds, 4-aminobenzamidine and 4-methyl-2-guanidinoquinazoline, failed to show complement inhibition (Table 3). These findings led us to suspect that these agents

* Although not detailed here, the amidines also failed to block the reversed passive Arthus reaction, which is also complement mediated.

Table 5. Volumes of distribution for basic compounds*

	Volume of distribution (l/kg)	Species	Reference
4-Aminobenzamidine 	2.7	Guinea pig	this paper
Bunamidine 	ca. 6	Dog	22
Guanethidine 	48 15	Rat Rat	23 24
Guanisoquin 	3.8	Dog	25
Pentamidine 	3	Man	26,27
Stilbamidine 	ca. 2	Mouse	28

*Computed from plasma concentrations reported in cited references.

were cleared from the blood so rapidly that an inhibitory concentration would not be maintained. Blood levels of 4-aminobenzamidine were therefore examined as a function of time. The pharmacokinetic study demonstrated that drug concentrations (*ca.* 0.3×10^{-3} M at zero time) were below the minimum *in vitro* inhibitory concentration, *ca.* 6×10^{-3} M. Not only did the drug undergo both very rapid and extensive extravascular redistribution, but the *ca.* 1.5 per cent of dose remaining disappeared from plasma with a mean half-life of only 4.3 min. Thus, sustained levels of 4-aminobenzamidine sufficient to inhibit vascular complement could not be achieved.

Distribution of compounds between tissue and plasma compartments appears to be governed by several structural and chemical characteristics. In general, acidic character and protein binding tend to confine drugs within the plasma. This behavior is exhibited by inter alia, acidic, non-steroidal antiinflammatory agents [19, 20], which show volumes of distribution of *ca.* 0.1 liter/kg, approximately the blood volume [21]. We have computed volumes of distribution from literature data [22–28] for pentamidine and other amidines and guanidines (Table 5). Such highly basic compounds appear to partition rapidly out of the central circulation and, hence, exhibit large distribution volumes (2–48 liters/kg). Thus, only very low blood concentrations are attained even after large parenteral doses of such drugs. None of the agents we examined had any physiochemical characteristics likely to alter the expected large volume of distribution. Similarly, none of the 108 compounds reviewed by Hansch and Yoshimoto [5] would be judged likely to distribute preferentially in the plasma. This also appears to be the case for most of the compounds referred to by Baker and Cory [29] as well as for the very active drug, *m*-[*m*-(*p*-nitrophyenlyureido)phenoxypropyloxy]benzamidine, reported by Glovsky *et al.* [8]. We believe that, by virtue of their intrinsic chemical properties, the amidine-like complement inhibitors synthesized to date will prove to be inactive *in vivo* since they will distribute rapidly out of the central compartment, and will probably undergo rapid excretion. We conclude that structural modifications such as introduction of acidic functionalities must be made to permit distribution in the plasma volume before *in vivo* anticomplementary activity can be expected.

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