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ANTI-TRYPANOSOMAL ACTIVITY OF CERTAIN PHENYLDIAZOAMINO- AND PHENYLAZOAMINO-PHENANTHRIDINIUM COMPOUNDS

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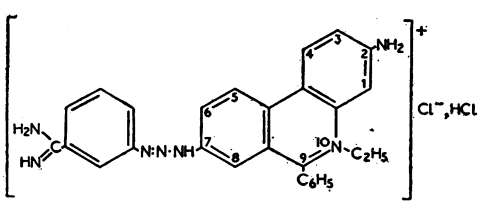
Red phenyldiazoaminophenanthridinium and purple phenylazoaminophenanthridinium compounds, most of which were obtained as isomeric pairs, were tested in mice for curative and prophylactic action against *Trypanosoma congolense*. Several of the individual compounds showed a high degree of activity, the red isomer of each pair usually being more active therapeutically and prophylactically than the purple one, although high prophylactic effect was not always associated with high therapeutic potency. The most interesting isomeric pair was that consisting of metamidium, 7-(*m*-amidinophenyldiazoamino)-2-amino-10-ethyl-9-phenylphenanthridinium chloride hydrochloride (red isomer) and *x*-(*m*-amidinophenylazo)-2,7-diamino-10-ethyl-9-phenylphenanthridinium chloride hydrochloride (purple isomer). Among the individual compounds, the one with the greatest curative action was the red isomer of metamidium, now known as isometamidium. Its 10-methyl homologue was slightly less active and its *p*-amidino-isomer was considerably less active. The compounds with the greatest prophylactic action were 2,7-di(*m*-amidinophenyldiazoamino)-10-ethyl-9-phenylphenanthridinium chloride dihydrochloride trihydrate, its 10-methyl homologue, and the red *m*-amidino-isomers mentioned above. The only other compounds to show appreciable prophylactic action were three guanidino-substituted phenyldiazoaminophenanthridines, although they were less active than metamidium at low doses. One of these, 2-amino-10-ethyl-7-(*m*-guanidinophenyldiazoamino)-9-phenylphenanthridinium chloride hydrochloride, was also very active therapeutically.

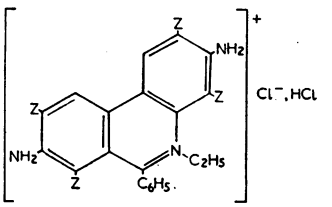
Several phenanthridinum compounds were shown by Browning, Morgan, Robb & Walls (1938), Wien (1946), Walls (1947), Browning (1949), Brownlee, Goss, Goodwin, Woodbine & Walls (1950) and Woolfe (1952, 1956) to possess considerable anti-trypanosomal activity. Of these, 2,7-diamino-10-methyl-9-phenylphenanthridinum bromide (dimidium bromide) and its 10-ethyl analogue (homidium bromide) have been used extensively as curative agents for bovine trypanosomiasis. More recently another phenanthridinium compound, 2-amino-7-(2-amino-6-methylpyrimid-4-ylamino)-9-(*p*-aminophenyl)-10-methylphenanthridine 10,1′-dimethobromide (Prothidium bromide), has been found to have a prophylactic effect superior to that of quinapyramine prophylactic B.Vet.C. (Woolfe & Watkins, 1956; Robson & Milne, 1957; Robson & Hope Cawdery, 1958; Smith, 1959).

In 1958 Wragg, Washbourn, Brown & Hill described the chemical and biological properties of metamidium, prepared by the condensation of *m*-amidinobenzenedia-

zonium chloride and homidium chloride. The product was obtained as a mixture of *red* and *purple* isomers, to which the structures 2-(*m*-amidinophenyldiazoamino)-7-amino-10-ethyl-9-phenylphenanthridinium chloride hydrochloride and 7-(*m*-amidinophenyldiazoamino) - 2 - amino - 10-ethyl-9-phenylphenanthridinium chloride hydrochloride (I) respectively were provisionally assigned. Recent chemical investigations (Berg, 1960) have shown that in fact the *red* isomer is represented by formula (I) and the *purple* isomer by formula (II). It will be noted that (I) contains the diazoamino structure in which the *m*-amidinophenylazo-group is linked to the aromatic amino-group, and (II) contains the amino-azo-system in which the amidinophenylazo-group is coupled directly to the aromatic ring at one of the positions *ortho* to the amino-groups.



I
ISOMETAMIDIUM



II
where one Z = *m*—NH_2·C(:NH)·C_6H_4·N:N—
and the others are H

The numbering of the phenanthridine nucleus in this paper corresponds to the system used in earlier papers on this series of trypanocidal compounds, but differs from the I.U.P.A.C. rules as set out in *Handbook for Chemical Society Authors, 1960.*

The present paper deals with the anti-trypanosomal effect of an analogous series of compounds (Washbourn & Wragg, in preparation ; Berg, in preparation) prepared by the coupling reaction of substituted benzenediazonium chlorides with homidium, dimidium and phenidium salts. Throughout this paper the expression " mixture " refers to the two isomers mixed in the proportions obtained from the coupling reaction between the requisite diazonium and phenanthridinium salts when this reaction is carried out under the conditions described. It does *not* refer to artificially prepared mixtures of the pure isomers.

METHODS

A laboratory strain of *T. congolense* (kindly supplied by Dr W. E. Ormerod), which had been maintained by intraperitoneal syringe-passage in mice over many years, was used in these experiments. It produced a subacute infection resulting in a variable parasitaemia, and the mice normally died within 1 to 3 weeks.

The subcutaneous LD50 of each compound was first determined approximately with 5 mice/dose, and some of the more active compounds were re-examined with 10 mice/dose to obtain a more accurate figure. It was not considered necessary to determine the limits of error of these estimations, and relative toxicities were approximately determined by the relationship of the LD50 figures.

Therapeutic experiments. Mice with an infection in the peripheral blood stream of 1 to 10 trypanosomes/high-power field were given a single subcutaneous injection. Five mice were used for each dose in the preliminary screening tests and 10/dose in the subsequent experiments

with the more active drugs. Wet blood smears were examined 3 times a week for 4 weeks, and the number of animals cleared of trypanosomes during that period (and therefore considered "cured") was noted. The mean curative dose (CD50) was then obtained either by inspection or graphically by de Beer's method (1945), and the approximate relative activities were determined by the relationship of the CD50 figures.

Prophylactic experiments. In these experiments a single subcutaneous injection of the substance was given to a number of mice, and at intervals groups of 6 or more were challenged intraperitoneally with *T. congolense* (approximately 65,000 trypanosomes/mouse). No mouse was challenged more than once. The mice were examined for trypanosomes for 4 weeks after challenge, as in the therapeutic experiments, and those which remained clear of trypanosomes for this period were considered to have been protected. Untreated controls became positive for trypanosomes within 5 days.

In the earlier toxicity, therapeutic and prophylactic experiments, homidium chloride (B.Vet.C.) was used as the reference compound, but later it was replaced by metamidium chloride.

RESULTS

For homidium, the LD50 ranged from 50 to 100 mg/kg and the CD50 from 0.03 to 0.2 mg/kg. To facilitate comparisons of potency of the compounds shown in Tables 2 to 5, their toxicity and activity have been expressed as homidium equivalents (homidium in the same experiment = 1). When metamidium was used as a standard of comparison, potencies were recalculated to give the homidium equivalent on the basis of metamidium being one-third as toxic and four times as active as homidium in a direct comparison (Table 1).

TABLE 1

DIRECT COMPARISON OF THE TOXICITY AND THERAPEUTIC ACTIVITY AGAINST *T. CONGOLENSE* IN MICE OF HOMIDIUM AND METAMIDIUM INJECTED SUBCUTANEOUSLY

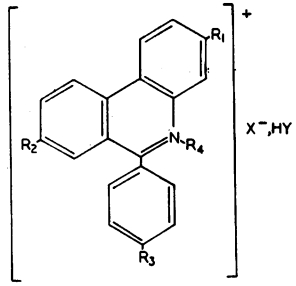
Compound	LD50 mg/kg	Limits of error % (P=0·05)	Slope "b"	CD50 mg/kg	Limits of error % (P=0·05)	Slope "b"
Homidium	75·0	85–115	6·1	0·03	73–137	4·1
Metamidium	210·0	84–119	7·0	0·0075	76–135	4·5

In the prophylactic experiments, except those shown in Table 6, groups of mice were challenged 3, 6, 9 or 12 weeks after dosing with 25 mg/kg. The prophylactic period given in the tables is that for which complete protection was provided. Homidium failed to protect mice after 3 weeks.

m- *and* p-*Amidinophenyl compounds.* All the compounds in this group were less toxic than homidium. This difference was not simply the effect of their generally lower solubility, because those compounds that were relatively soluble were still only about one-third as toxic as the standard compound. The red isomer of each pair was more active than the purple one. Also, greater therapeutic activity resulted from *m*-amidino- than from *p*-amidino-substitution and from 10-ethyl than from 10-methyl quaternation. Maximum activity was found in the red *m*-amidino-10-ethyl isomer (M&B 4180, the bromide of isometamidium) (Table 2), which was about 14 times as active as homidium. M&B 5853 (Table 2), the deamino-analogue

TABLE 2

THE ACTIVITY OF SOME PHENYLDIAZOAMINOPHENANTHRIDINIUM SALTS (*RED* ISOMERS) AGAINST *T. CONGOLENSE* IN MICE



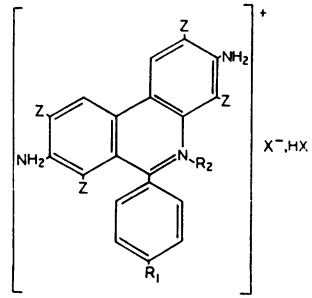
The doses and homidium equivalents are given in terms of the salts (cation content 70–83·5%). M&B 4180 is the bromide of isometamidium. M&B 4616 and M&B 4492 were obtained by the coupling reaction of diazotized 7-acetamido-2-amino-10-methyl (*or* ethyl)-9-phenylphenanthridinium chloride and *m*-aminobenzamidine monohydrochloride. M&B 4474 may equally well have the isomeric structure in which R_2 and R_3 are interchanged. This orientation has not been investigated. M&B 4762 is a mixture of two isomers, one with the structure given below, the other in which R_2 and R_3 are interchanged

(*a*)=*p*-NH_2.C(:NH).C_6H_4.N:N.NH–. (*b*)=CH_3.CO.O–. (*c*)=*m*-NH_2.C(:NH).C_6H_4.N:N.NH–

M&B no.	R_1	R_2	R_3	R_4	X	Y	Solubility w/v water	Relative toxicity (homidium=1)	Relative activity (homidium=1)	Protection (in weeks) afforded by 25 mg/kg subcutaneously
4261	$-NH_2$	*p*-NH_2.C(:NH).C_6H_4.N:N.NH–	H–	C_2H_5–	Cl	Cl	18%	0·3	0·05	<3
4180	$-NH_2$	*m*-NH_2.C(:NH).C_6H_4.N:N.NH–	H–	C_2H_5–	Br	Br	0·7%	<0·25	14·0	>12
5853	–H	*m*-NH_2.C(:NH).C_6H_4.N:N.NH–	H–	C_2H_5–	Cl	Cl	2·0%	0·3	0·7	3
4492	*m*-NH_2.C(:NH).C_6H_4.N:N.NH–	CH_3.CO.NH–	H–	C_2H_5–	Cl	Cl	0·6%	<0·1	0·002	Not tested
4616	*m*-NH_2.C(:NH).C_6H_4.N:N.NH–	CH_3.CO.NH–	–H	CH_3–	Cl	Cl	0·5%	0·1	<0·001	Not tested
4415	$-NH_2$	*m*-NH_2.C(:NH).C_6H_4.N:N.NH–	–H	CH_3–	Br	Br	<0·1%	<0·02	12	>9
4474	–H	$-NH_2$	(*a*)	CH_3–	Cl	(*b*)	<0·1%	<0·1	<0·001	Not tested
4762	–H	$-NH_2$	(*c*)	CH_3–	Cl	Cl	0·3%	1·0	0·005	<3
4814	$-NH_2$	*p*-NH_2.C(:NH).NH.N:CH.C_6H_4.N:N.NH–	–H	C_2H_5–	Cl	Cl	4·0%	0·03	0·02	12
4907	$-NH_2$	*m*-NH_2.C(:NH).NH.N:CH.C_6H_4.N:N.NH–	–H	C_2H_5–	Cl	Cl	2·5%	<0·1	0·01	12
4806	$-NH_2$	*m*-NH_2.C(:NH).NH.C_6H_4.N:N.NH–	–H	C_2H_5–	Cl	Cl	2·0%	0·1	2·0	6

TABLE 3

THE ACTIVITY OF SOME AMIDINOPHENYLAZOPHENANTHRIDINIUM SALTS (*PURPLE* ISOMERS) AGAINST *T. CONGOLENSE* IN MICE



where one Z=amidinophenylazo and the others are H

The doses and homidium equivalents are given in terms of the salts (cation 70–83·5%)

M&B no.	R_1	R_2	Z	X	Solubility w/v water	Relative toxicity (homidium=1)	Relative activity (homidium=1)	Protection (in weeks) afforded by 25 mg/kg subcutaneously
3989	H–	C_2H_5–	*p*-NH_2.C(:NH).C_6H_4.N:N–	Cl	0·3%	0·1	0·025	<3
4250	H–	C_2H_5–	*m*-NH_2.C(:NH).C_6H_4.N:N–	Br	0·7%	0·13	1·0	<4
3813	H–	CH_3–	*p*-NH_2.C(:NH).C_6H_4.N:N–	Cl	1%	0·1	0·01	<3
4418	H–	CH_3–	*m*-NH_2.C(:NH).C_6H_4.N:N–	Cl	>5%	0·25	0·5	<3
4669	NO_2–	CH_3–	*m*-NH_2.C(:NH).C_6H_4.N:N–	Cl	3%	0·3	0·1	3

of the red isomer, was slightly less active than homidium. M&B 4616 and 4492 (Table 2), the 7-acetamido-2-(*m*-amidinophenyldiazoamino)-analogues of dimidium and homidium respectively, like the phenidium derivatives, M&B 4474 and 4762 (Table 2), were very much less active than homidium.

The prophylactic activity of M&B 4634 (Tables 4 and 6) was slightly superior at 25 mg/kg to that of metamidium. In both these mixtures the prophylactic activity was due primarily to the red isomer, the purple one being virtually inactive. Equally active prophylactically was M&B 4596 (Tables 5 and 6), 2,7-di(*m*-amidinophenyldiazoamino)-10-ethyl-9-phenylphenanthridinium chloride dihydrochloride trihydrate, although it was considerably less effective therapeutically (Berg, Brown, Hill & Wragg, 1961). A similar result was obtained with the corresponding 10-methyl derivative (M&B 5657).

TABLE 4

THE ACTIVITY OF M&B 4634 AND METAMIDIUM (MIXED ISOMERS) AGAINST *T. CONGOLENSE* IN MICE

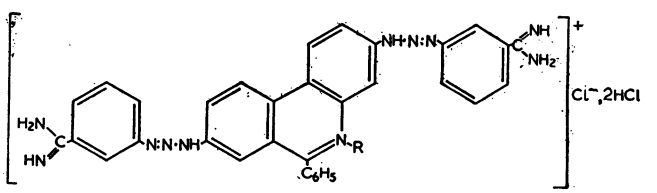
The doses and homidium equivalents are given in terms of the salts (cation contents 75–77·7%). M&B 4180A (isometamidium) and 4250A are the chlorides of M&B 4180 and 4250 respectively. M&B 4634 is the 10-methyl quaternary homologue of metamidium

Compound	Composition	Solubility w/v water	Relative toxicity (homidium=1)	Relative activity (homidium=1)	Protection (in weeks) afforded by 25 mg/kg subcutaneously
M&B 4634	M&B 4415 (Table 1) 40–45% M&B 4418 (Table 2) 55–60%	5%	0·3	2·0	20
Metamidium (M&B 4404)	M&B 4180A 40–45% M&B 4250 50–60%	12·5%	0·3	4·0	16

TABLE 5

THE ACTIVITY OF TWO DI(*m*-AMIDINOPHENYLDIAZOAMINO)PHENANTHRIDINIUM SALTS AGAINST *T. CONGOLENSE* IN MICE

The doses and homidium equivalents are given in terms of the salts (cation contents 70·8–71%)



M&B no.	R	Solubility w/v water	Relative toxicity (homidium=1)	Relative activity (homidium=1)	Protection (in weeks) afforded by 25 mg/kg subcutaneously
5657	Me	2%	<0·05	0·01	12
4596	Et	1·5%	<0·1	0·05	>24

Halogen substitution in the amidinophenyl ring. The introduction of a chlorine atom in the *meta*-position to the amidino-group of metamidium reduced the therapeutic activity somewhat, although the compound still had a better therapeutic

TABLE 6

DIRECT COMPARISON OF THE PROPHYLACTIC ACTIVITY AGAINST *T. CONGOLENSE* IN MICE OF METAMIDIUM WITH M&B 4596, M&B 4634, M&B 4814 AND M&B 4907

The formulae of these compounds are given in Tables 3, 4, 3, 1 and 1 respectively

	Dose injected subcutaneously		No. of mice protected/no. treated. Weeks after dosing										
Compound	mg/kg	mg (cation)/kg	2	3	4	6	8	9	12	16	20	24	26
Metamidium	25	20		6/6		5/5		6/6	11/11	10/10	11/14	8/10	
	5	4			4/4			4/6					
	1	0·8	6/6		19/19	9/14							
M&B 4596	25	18		6/6		5/5		6/6	20/20	19/21	15/15	10/10	
	5	3·6			4/5			4/4					
	1	0·7	5/6		11/19	9/16							
Metamidium	25	20								10/11	6/10		0/5
	5	4			4/4			4/6					
	1	0·8	6/6		5/5			0/3					
M&B 4634	25	19								5/5	12/12		9/10
	5	3·8			6/6			6/6					
	1	0·75	4/4		3/6			0/4					
Metamidium	25	20							6/6	9/10	4/6	0/7	
	5	4			6/6		5/5						
M&B 4814	25	20							5/5	6/8	1/4		
	5	4			0/6		0/8						
M&B 4907	25	20							10/10	8/10	3/3	1/6	
	5	4			0/6		0/8						

ratio than homidium, and decreased prophylactic activity drastically so that the compound failed to protect mice for 3 weeks. The relative proportion of red and purple isomers in this mixture was similar to that in metamidium.

Basic substituents other than an amidino-group. Of three guanidino-substituted compounds (Table 2), M&B 4806, with a 7-*m*-guanidinophenyldiazoamino-substituent, was the most active therapeutically. It was, however, less curative than the corresponding *m*-amidino-compound, M&B 4180, and also less prophylactic. The analogous *m*-guanidinoiminomethylphenyl derivative (M&B 4907, Table 2) had a much lower therapeutic activity but increased prophylactic effect, while the *p*-guanidinoiminomethylphenyl derivative (M&B 4814) was as active but more prophylactic than the *p*-amidino-compound, M&B 4261 (Table 2). M&B 4814 is outstanding in being the only *para*-substituted compound to show any great degree of prophylactic activity.

2,7- Diamino-*x*- (4 - dimethylaminophenylazo) -10-ethyl-9-phenylphenanthridinium methobromide bromide and 2,7-diamino-*x*-(4-diethylaminoethoxycarbonylphenylazo)-10-ethyl-9-phenylphenanthridinium chloride hydrochloride were much less active therapeutically than homidium.

Sulphamoyl, carbamoyl and nitro-substitution. Replacing the *p*-amidino-group in M&B 4261 (Table 2) and in M&B 3989 (Table 3) by a sulphamoyl or carbamoyl group had little influence on therapeutic activity; replacing it by a chlorine atom or by a carboxyl group reduced the therapeutic activity. Replacement of the *m*-amidino-group in the isomers of metamidium by a sulphamoyl or carbamoyl group resulted in a marked reduction of therapeutic activity to a value below that

for the corresponding *para*-substituted compounds. This applied also to the prophylactic activity of the red *m*-sulphamoyl isomer. Replacement of the *m*-amidino-group in the metamidium isomers by a nitro-group also reduced therapeutic activity. 2-Amino-7-(*o*-chlorophenyldiazoamino)-10-ethyl-9-phenylphenanthridinium chloride and the corresponding purple isomer were less active than homidium.

DISCUSSION

The general conclusions which may be drawn from this work are (*a*) that an amidino-group was superior to any of the other substituents which were tried, (*b*) that *meta*-substitution was better than *para*-substitution, and (*c*) that the red phenyldiazoamino-isomers were more active than the purple phenylazoamino- ones.

Of all the compounds tested, seven combined some curative action with a considerable degree of prophylactic activity. They were the bromide of isometamidium (M&B 4180) and its methyl quaternary homologue (M&B 4415), the 2,7-disubstituted analogue of metamidium (M&B 4596) and its methyl quaternary homologue (M&B 5657), and three guanidines listed in Table 2. Of these seven, the first pair were outstanding, and mixed with their corresponding purple isomers, to give metamidium and M&B 4634 respectively, they maintained a high curative and prophylactic potency.

Comparative tests showed that a subcutaneous dose of 25 mg/kg of M&B 4634 gave more prolonged protection that the same dose of metamidium, in spite of the fact that M&B 4634 was slightly less active therapeutically than metamidium. With M&B 4596, 4814 and 4907 the contrast was even greater since they were no less protective than metamidium at 25 mg/kg but were very much less active therapeutically. Because of this prolonged protection they must be intrinsically very active compounds. A possible explanation of their poor therapeutic effect is that they are fixed in the host's tissues more rapidly than metamidium, before their full anti-trypanosomal effect is manifested.

Isometamidium (M&B 4180A) and M&B 4596 are now under test in African cattle. The results so far are very encouraging. The toxicity and activity in African cattle of metamidium (M&B 4404) have been reported by Fairclough (1958), Whiteside (1958, 1960), Fiennes (1960), Kirkby (1960), Smith & Brown (1960), Stephen (1960) and others. The local tolerance of cattle to subcutaneous injections is poor; intramuscular injections are better tolerated, however, and satisfactory results have been recorded in most instances where doses of 3 mg/kg or less have been used. An exception exists in the case of Northern Nigerian cattle, which often react severely to relatively small doses. Systemic tolerance is generally satisfactory, provided a dose of 3 mg/kg is not exceeded, although here again exceptions have been recorded, notably amongst Nigerian Fulani cattle. The reasons for the exceptionally poor tolerance of Northern Nigerian cattle to metamidium and to certain other phenanthridines are not understood.

Experiments in East and West Africa showed metamidium to be considerably more active curatively against *T. congolense* than homidium, and data from trials conducted in several localities suggest that a dose of 0.5 to 1.0 mg/kg may be relied upon to eliminate most of the trypanosomes encountered under field conditions.

In a heavy challenge (*T. congolense, T. vivax* and *T. brucei*) in East Africa an intramuscular dose of 4 mg/kg protected cattle for an average of 18 weeks, compared with 10 weeks for 11.7 mg/kg of quinapyramine (prophylactic) in the same trial (Smith & Brown, 1960). In West Africa one of seven Fulani cattle given 3 mg/kg succumbed to a natural challenge (*T. congolense* and *T. vivax*) after 15 weeks (Kirkby, 1960).

In laboratory tests with cattle (Whiteside, 1960), metamidium has been found to cure strains of *T. congolense* resistant to quinapyramine, homidium, dimidium, Prothidium and Berenil, and therefore has great value as a "sanative." Strains resistant to metamidium could be cured satisfactorily with Berenil.

We are very grateful to our colleagues, Mr S. S. Berg, Mr L. Bretherick, Mr K. Washbourn and Dr W. R. Wragg for supplying the compounds, and to Mr C. G. L. Beveridge for compiling the summary of the field trials with metamidium. We also wish to thank our colleagues, Dr J. N. Ashley, Dr H. J. Barber and Dr R. Wien, for their helpful discussions, and Miss G. Merchant and Mr T. Mercer for technical assistance.

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