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INHIBITORS OF HISTAMINE METABOLISM *IN VITRO* AND *IN VIVO*

CORRELATIONS WITH ANTITRYPANOSOMAL ACTIVITY

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Abstract—The effects of antimalarial and antitrypanosomal drugs on the activity of histamine *N*-methyl transferase and diamine oxidase *in vitro*, as well as diamine oxidation and histamine levels *in vivo*, were examined. Diamidine antitrypanosomal drugs which interfere with polyamine metabolism were found to be potent inhibitors both *in vitro* and *in vivo*. Antrycide (quinapyramine) and isometamidium were the best inhibitors of both enzymes. K_i values for histamine *N*-methyl transferase were 3×10^{-8} M for both compounds, and the inhibition was competitive for histamine. Antrycide and isometamidium were both non-competitive inhibitors of diamine oxidase, having K_i values of 6×10^{-9} M and 3×10^{-9} M respectively. Isometamidium elevated histamine levels in rat kidney 2-fold and produced a long-term inhibition of putrescine oxidation *in vivo*. Among the compounds examined, only known active antitrypanosomal agents inhibited both histamine *N*-methyl transferase and diamine oxidase *in vitro* as well as putrescine oxidation *in vivo*. These observations suggest that the enzymes acting on histamine and putrescine as substrates can be used to select compounds which interfere with polyamine metabolism and that persistence of such compounds *in vivo*, as indicated by inhibition of putrescine oxidation, correlates with favorable chemotherapeutic properties as antitrypanosomal agents.

In mammalian species, histamine (HA)§ is metabolized either by methylation of the nitrogen in the imidazole ring [1] in an *S*-adenosylmethionine-dependent reaction catalyzed by histamine *N*-methyl transferase (HMT) or by oxidative deamination [2] using the enzyme diamine oxidase (DAO), also known as histaminase. Putrescine, the diamine precursor of polyamines, is also metabolized by DAO [3]. The distribution of these enzymes is both tissue and species dependent.

Inhibition of HMT has been reported for representatives of several classes of drugs, including antimalarials, anticancer agents, antihistamines, diuretics and local anesthetics [4–8]. Drugs within each class varied considerably in potency. Structure–activity studies on the inhibition of HMT [9] indicate that a wide variety of nitrogen-containing heterocycles effectively inhibit the enzyme. These observations, coupled with the findings that several of these compounds interact with the histamine H_2 receptor [10, 11], led to the further investigation of the effects of these compounds on the activity of DAO. Several lipid-soluble inhibitors of dihydrofolate reductase were found to inhibit HMT and some of these compounds also elevated HA levels *in vivo*. Many of the compounds which inhibit HMT are also potent inhibitors of DAO [4, 12]. Ma and Sourkes [13] showed that the antimalarial drugs amodiaquine, quinacrine and chloroquine inhibit DAO both *in vitro* and *in vivo*, amodiaquine being the most potent of the three in both respects. Although amodiaquine inhibits HMT *in vivo*, as evidenced by the inhibition of the metabolism of administered [^{14}C]HA [14], endogenous levels of HA are unaffected [15]. Blaschko *et al.* [16, 17] also demonstrated that DAO is inhibited *in vitro* by several straight-chain and aromatic diguanidines. Among the compounds studied were the antitrypanosomal drugs propamidine, pentamidine and stilbamidine.

Because of the wide-ranging effects of numerous nitrogen-containing heterocycles on HA metabolism, further studies on the effects of antimalarial drugs on HMT and DAO were undertaken. Due to the early reports of Blaschko and coworkers [16, 17], the present studies were expanded to include the diamidines having antitrypanosomal activity. In this study the effects of these compounds on HMT and DAO *in vitro* and putrescine oxidation and HA levels *in vivo* were determined.

MATERIALS AND METHODS

Chemicals and drugs. [Methyl-^{14}C]*S*-Adenosylmethionine (57.8 mCi/mmole), [methyl-^{3}H]*S*-adenosylmethionine (10.5 Ci/mmole) and [1,4-^{14}C]putrescine (96.4 mCi/mmole) were purchased from the New England Nuclear Corp.; histamine, putrescine, aminoguanidine, *S*-adenosylmethionine, chloroquine, quinacrine, ethidium and acriflavin were from

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§ Abbreviations used: HA, histamine; HMT, histamine *N*-methyl transferase; DAO, diamine oxidase; SAM, *S*-adenosylmethionine; MGBG, methylglyoxal bis(guanylhydrazone); and trimetrexate (TMQ, 2,4-diamino-5-methyl-6-[(3,4,5-trimethoxyanilino)methyl]quinazoline).

the Sigma Chemical Co.; and methylglyoxal bis-(guanylhydrazone) was from the Aldrich Chemical Co. Amicarbalide, isometamidium and pentamidine were gifts from May & Baker; Antrycide was from Imperial Chemical Industries; Berenil (diminazene aceturate) from Farbwerke Hoechst AG; amodiaquine from Parke-Davis; primaquine and hydroxychloroquine from Sterling-Winthrop; Bayer b 1694 from Bayer AG; WR 199-385 from the Walter Reed Army Institute of Research; and trimetrexate (TMQ, 2,4-diamino-5-methyl-6-[(3,4,5-trimethoxyanilino)methyl]quinazoline, NSC 249008) from the Drug Development Branch of the National Cancer Institute. Imidocarb was synthesized at the Burroughs Wellcome Co.

Enzyme preparation and assays. HMT was partially purified from the cerebral cortex of bovine brain, using the ammonium sulfate and dialysis steps described by Brown *et al.* [18]. Reaction mixtures for HMT consisted of 10 μmoles sodium phosphate, pH 7.4, 15 nmoles histamine, 14.2 nmoles [^{14}C]SAM, enzyme and, where appropriate, inhibitor, in a total volume of 300 μl. The reaction was initiated by the addition of [^{14}C]SAM and incubated for 15 min at 37°. The reaction was terminated by the addition of 0.5 ml of 0.5 M borate buffer, pH 11, and the radioactive product was extracted with 6 ml of toluene–isoamyl alcohol (1:1). Four milliliters of the organic extract was counted in 10 ml of an Omnifluor–toluene scintillation mixture containing 2 ml of absolute ethanol.

DAO was partially purified from rat cecum. Ceca from twenty rats were removed, washed in physiological saline, and homogenized in 4 vol. of 0.05 M sodium phosphate, pH 7.2, with a Polytron homogenizer. The homogenate was then centrifuged at 26,000 *g* for 20 min. The supernatant was fractionated with ammonium sulfate, and the fraction precipitating between 25 and 60% ammonium sulfate was retained for further purification. Following dialysis of the ammonium sulfate precipitate, the preparation was charged onto a 2.5 × 25 cm column of DEAE-cellulose previously equilibrated with 0.01 M sodium phosphate, pH 7.2, washed with the same buffer, and eluted with 0.01 M sodium phosphate, pH 7.2, using a linear gradient from 0 to 0.5 M NaCl. The active fractions were pooled, concentrated, and used as the source of enzyme. DAO was assayed using the method of Okuyama and Kobayashi [19]. Incubation mixtures consisted of 100 μmoles sodium phosphate buffer, pH 7.2, 0.2 μmoles putrescine (0.1 μCi [1,4-^{14}C]putrescine), enzyme and, where appropriate, inhibitor, in a total volume of 1 ml. Incubations were for 20 min at 37°. The reactions were terminated by adding 1 ml of 0.5 M borate buffer, pH 10.5, and 20 μl of 0.01 M aminoguanidine, and the radioactive product was extracted with 6 ml of toluene. Four milliliters of the toluene extract was counted in 10 ml of an Omnifluor–toluene scintillation mixture.

Putrescine oxidation *in vivo* was determined essentially as described by Pegg and McGill [20]. Male Sprague–Dawley rats were dosed i.p. with drug in 0.9% (w/v) saline. Control rats received only saline. At selected times after administration of drug, [^{14}C]putrescine (20 mg/kg, 2 μCi/animal) was injected i.p., and the animals were placed in glass metabolic cages through which a constant stream of air was pumped. The air exiting the metabolic cage was passed through two CO_2 trapping chambers each containing 200 ml of 1 N NaOH. At various times 1-ml samples from each chamber were taken and counted in 15 ml Aquasol.

Kinetic analysis. Kinetic analysis was carried out using the method of Lineweaver and Burk [21]. K_i values were calculated from replots as described by Segal [22].

Analysis of histamine levels. The effects of the drugs on HA levels in kidney were determined in male Sprague–Dawley rats (180–200 g). Drugs were administered i.p. HA levels were determined using the enzymatic assay of Taylor and Snyder [23] as modified by Beaven *et al.* [24]. Tissue extracts were chromatographed on Dowex 50 to remove drug which would interfere with the enzymatic assay.

Antitrypanosomal activity. The EATRO 110 isolate of *Trypanosoma b. brucei* was used in this study. Infection with this monomorphic strain is fatal in 3–6 days. Groups of five mice were infected i.p. with 2.5 to 5.0 × 10^5 trypanosomes. Drug treatment was begun 24 hr after infection and given once daily for 3 successive days. Survival was measured as the average survival in days beyond the death of the control animals. A drug was considered curative when the treated animals survived 30 days beyond the infected controls, with no evidence of parasites in peripheral blood smears.

RESULTS

The results presented in Table 1 show that most of these drugs are very effective inhibitors of HMT. Among the antimalarials, only primaquine, an 8-aminoquinoline derivative, was relatively inactive as an inhibitor of the enzyme. Chloroquine, hydroxychloroquine and amodiaquine, all substituted 4-aminoquinolines, as well as quinacrine, a substituted 9-aminoacridine, were all potent inhibitors of the enzyme. The antitrypanosomal drugs studied also showed marked inhibition of HMT. Included in this list are the babesicides amicarbalide and imidocarb which Nathan *et al.* [25] have shown to be extremely effective in curing *T. b. brucei* infections in mice. MGBG and aminoguanidine are also included since both have been shown previously to be effective inhibitors of DAO *in vitro* and *in vivo* [20] and MGBG exhibited weak and transitory activity against *T. b. brucei* [25].

Table 2 illustrates the effects of the antimalarial and antitrypanosomal drugs on the activity of DAO *in vitro*. Among the antimalarials, primaquine was a relatively poor inhibitor of DAO; the remaining antimalarials as well as the antitrypanosomal drugs were good inhibitors of this enzyme. Isometamidium, prothidium, Antrycide and imidocarb were all extremely potent inhibitors of the enzyme. K_i values for selected compounds are presented in Table 3. All of the drugs examined were competitive inhibitors (vs HA) of HMT. With DAO, inhibition by all of the compounds except aminoguanidine was non-competitive with respect to the amine substrate. Inhibition by aminoguanidine was competitive.

Table 1. Inhibition of HMT by antimalarial and antitrypanosomal drugs

	Percent inhibition			
	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M
Antimalarials				
Primaquine	4	0		
Chloroquine	93	56	0	
Hydroxychloroquine	90	61	11	
Amodiaquine	100	96	81	32
Quinacrine	98	83	18	0
Antitrypanosomals				
Ethidium	97	76	25	
Isometamidium	100	97	76	15
Prothidium	98	81	45	
Pentamidine	99	72	23	
Acriflavin	96	73	12	
Berenil	99	6	0	
Antrycide	100	88	40	0
Bayer b 1694	93	64	0	
WR 199-385	66	33	11	
Other				
Imidocarb*	98	92	57	15
Amicarbalide*	95	78	45	
MGBG*	31	0		
Aminoguanidine	24	0		

* Antitrypanosomal activity has been demonstrated in animal models.

Of primary importance is whether or not compounds which inhibit HMT *in vitro* will also inhibit the enzyme *in vivo* with the consequent elevation of tissue HA levels. Earlier studies on the effects of several inhibitors of HMT on HA levels in brain [26, 27] showed that the compounds studied had only weak and transitory activity *in vivo*. It was later demonstrated [4, 5, 12] that metoprine and trimetrexate, two lipid-soluble antifolates which are potent inhibitors of HMT *in vitro*, can also markedly elevate HA levels in rat brain and kidney. Thus, the effects of two of the most potent inhibitors of HA metabolism *in vitro*, isometamidium and imidocarb, were studied to determine their effects on HA levels *in vivo*. As seen in Fig. 1, both compounds elevated HA levels in rat kidney. Isometamidium produced approximately a 2-fold elevation which reached its maximum 1 hr after administration of the drug. Kidney levels were still substantially elevated 24 hr after administration. In contrast, imidocarb elevated HA levels 15-fold 30 min after administration. HA levels then decreased and approached normal levels 24 hr after administration.

Since the compounds studied were potent inhibi-

Table 2. Inhibition of DAO by antimalarial and antitrypanosomal drugs

	Percent inhibition					
	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M
Antimalarials						
Primaquine	53	2	0			
Chloroquine		76	49	10		
Hydroxychloroquine		67	31	1		
Amodiaquine		96	90	63		
Quinacrine		92	78	25		
Antitrypanosomals						
Ethidium	88	66	12			
Isometamidium			100	99	78	11
Prothidium				100	78	32
Pentamidine	96	93	51			
Acriflavin	94	83	32			
Berenil		98	91	49	7	
Antrycide		100	99	93	59	0
Bayer b 1694		97	95	43	0	
WR 199-385	93	82	52	8		
Other						
Imidocarb		98	94	62	8	
Amicarbalide	100	89	55	9		
MGBG		98	73	29		
Aminoguanidine		98	78	16		

Table 3. K_i values of antimalarial and antitrypanosomal drugs as inhibitors of HMT and DAO

	K_i ($\times 10^7$ M)	Type*
HMT		
Amodiaquine	0.52	C
Isometamidium	0.35	C
Antrycide	0.30	C
Imidocarb	1.0	C
Amicarbalide	5.5	C
DAO		
Amodiaquine	0.50	NC
Isometamidium	0.03	NC
Antrycide	0.06	NC
Imidocarb	0.32	NC
Amicarbalide	5.2	NC
MGBG	2.0	NC
Aminoguanidine	4.5	C

* C, competitive inhibition; NC, non-competitive

tors of HA metabolism *in vitro*, and the studies with isometamidium and imidocarb indicated that these compounds were capable of elevating HA levels *in vivo*, the effects of selected compounds on the oxidation of putrescine *in vivo* were determined. Figure 2 illustrates the effects of the antitrypanosomal drugs isometamidium, imidocarb, Antrycide and amicarbalide on the release of $^{14}CO_2$ from [1,4-^{14}C] putrescine. All four compounds substantially inhibited the enzyme initially. $^{14}CO_2$ release was still markedly inhibited 22 hr after administration of the drugs, with isometamidium proving to be the most effective inhibitor. $^{14}CO_2$ production was still 26% inhibited 41 hr after administration of the drug.

Figure 3 illustrates the effects of three other drugs, aminoguanidine, amodiaquine and trimetrexate, on the release of $^{14}CO_2$. Aminoguanidine was found to be a potent inhibitor of putrescine oxidation *in vivo*. Seventeen hours after administration, $^{14}CO_2$ production was only 11% of control. Similar results for

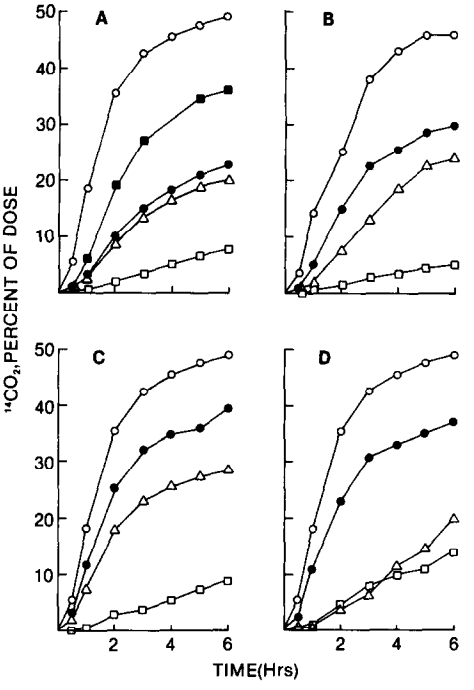

Fig. 2. Inhibition of putrescine oxidation *in vivo* by antitrypanosomal drugs. Rats were given a single i.p. injection of the antitrypanosomal drug in physiological saline. After appropriate time intervals, indicated below for each drug, [1,4-^{14}C]putrescine (20 mg/kg; 2 μCi) was administered i.p. Control animals received physiological saline. Expired $^{14}CO_2$ was collected and counted as described in Materials and Methods. The cumulative exhalation of $^{14}CO_2$ is given as the percentage of the [^{14}C]putrescine administered. Key: (A) Isometamidium, 20 mg/kg; (□—□) 1 hr, (△—△) 17 hr, (●—●) 22.5 hr, (■—■) 41 hr, and (○—○) control. (B) Imidocarb, 50 mg/kg; (□—□) 1 hr, (△—△) 17 hr, (●—●) 23.5 hr, and (○—○) control. (C) Antrycide, 10 mg/kg; (□—□) 1 hr, (△—△) 17 hr, (●—●) 23 hr, and (○—○) control. (D) Amicarbalide, 50 mg/kg; (□—□) 1 hr, (△—△) 17 hr, (●—●) 25 hr, and (○—○) control.

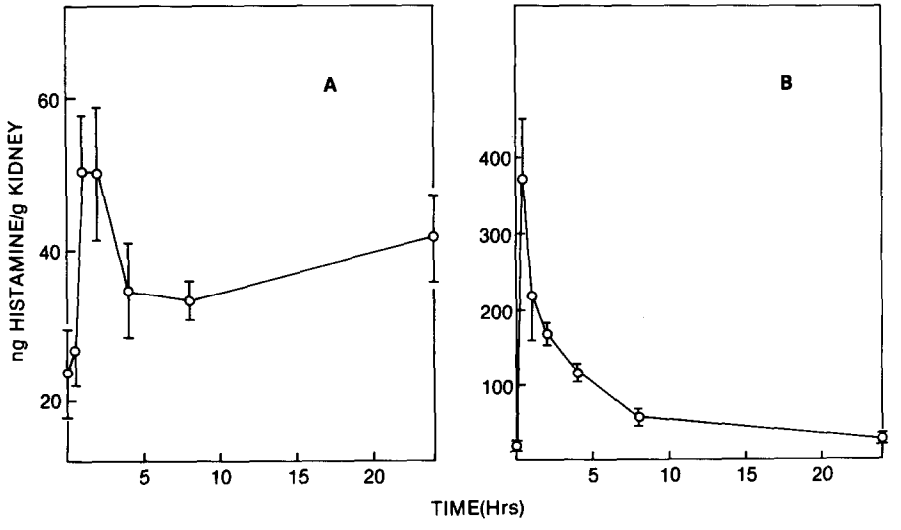

Fig. 1. Effects of isometamidium and imidocarb on histamine levels in rat kidney. (A) Isometamidium, 10 mg/kg. (B) Imidocarb, 50 mg/kg. Drugs were administered i.p. in saline. Each point represents the mean ± S.E.M. of five rats.

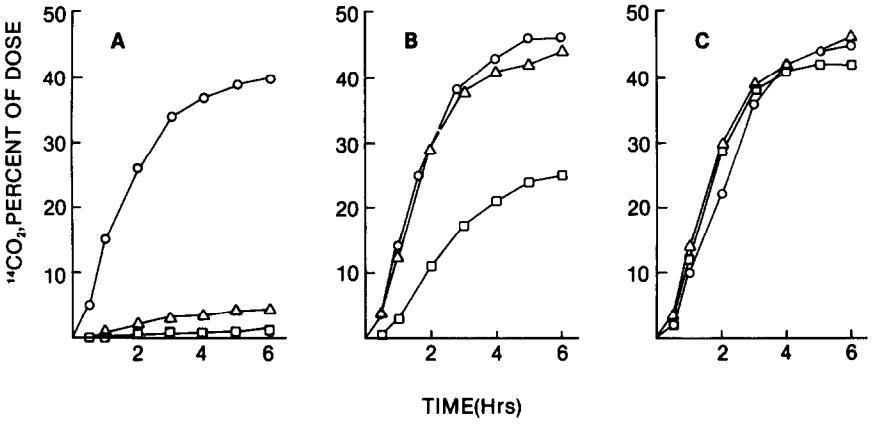

Fig. 3. Effects of drugs on the oxidation of putrescine *in vivo*. Rats were treated and $^{14}CO_2$ was measured as described in the legend to Fig. 2. Key: (A) Aminoguanidine, 50 mg/kg; (○—○) control, (□—□) 1 hr, and (△—△) 17.5 hr. (B) Amodiaquine, 50 mg/kg; (○—○) control, (□—□) 1 hr, and (△—△) 17 hr. (C) Trimetrexate, 40 mg/kg; (○—○) control, (□—□) 2 hr, and (△—△) 22 hr.

Table 4. Activity of trypanocides and other agents on experimental *Trypanosoma brucei* infections in mice*

Drug	Dose (mg/kg)	Activity
Isometamidium	1.0	Curative†
Antrycide	2.5	Curative
Amicarbalide	10.0	Curative
Imidocarb	5.0	Curative
Berenil	2.5	Curative
Pentamidine	1.0	Curative
Prothidium	2.0	Curative
Bayer b 1694	5.0	Curative
MGBG	75	Partially protective‡
Amodiaquine	50	No effect
Aminoguanidine	25	No effect
Trimetrexate	35	No effect

* Groups of five mice were infected with 2.5 to 5.0 × 10^5 trypanosomes. Drug treatment was begun 24 hr after infection. Drugs were administered i.p., once daily, for 3 successive days.

† The drug was considered curative when treated animals survived 30 days beyond the infected controls.

‡ MGBG completely cleared the peripheral circulation of trypanosomes for 3–4 days, but animals relapsed and died within 2 weeks.

aminoguanidine and MGBG were found by Pegg and McGill [20]. In contrast, trimetrexate, with a K_i vs DAO of 4.1 μM [12], and amodiaquine, though both good inhibitors of DAO *in vitro*, were essentially without effect on the activity of the enzyme *in vivo*. Amodiaquine produced less than 50% inhibition 1 hr after drug administration, and trimetrexate had no effect on $^{14}CO_2$ release even 2 hr after the drug was given. Thus, though both compounds are quite active as inhibitors of DAO *in vitro*, this activity is not observed *in vivo*.

The effects of several of these drugs on the course of *T. b. brucei* infection in mice are presented in Table 4. As can be seen, several standard trypanocides, as well as imidocarb and amicarbalide, cured *T. b. brucei* infection in mice. MGBG had only weak and transitory activity, and aminoguanidine, amodiaquine and trimetrexate had no effect on the course of the infection.

DISCUSSION

One purpose of this investigation was to correlate inhibition of HA- and putrescine-metabolizing enzymes *in vitro* with extent and duration of inhibition *in vivo* as indicated by the level of HA in tissues and by formation of carbon dioxide from putrescine. Many of the drugs examined were quite potent inhibitors of both HMT and DAO *in vitro*. Of the antimalarial, antitrypanosomal and anticancer drugs studied, isometamidium and Antrycide were the most active. K_i values for HMT were approximately 3×10^{-8} M for both compounds, placing them among the most potent inhibitors of HMT found thus far. Only the antifolates 2,4-diamino-5-(1-adamantyl)-6-methylpyrimidine (DAMP) and trimetrexate, with K_i values of 5×10^{-8} M and 7×10^{-9} M, respectively, were comparable [12]. All of these compounds were competitive inhibitors of HMT, with respect to HA. Antrycide and isometamidium were both non-competitive inhibitors of DAO, having K_i values of 6×10^{-9} M and 3×10^{-9} M respectively. With the exception of aminoguanidine which was a competitive inhibitor, the remaining compounds examined in this study, as well as in an earlier study [12] were non-competitive inhibitors of DAO. MGBG and aminoguanidine, though both very good inhibitors of DAO, were essentially inactive as inhibitors of HMT.

During examination of the data, it became apparent that only the active antitrypanosomal agents were potent inhibitors of HMT and DAO *in vitro* as well as putrescine oxidation *in vivo*. Amodiaquine, the most active antimalarial *in vitro*, was relatively inactive *in vivo*. At a dose of 50 mg/kg, the oxidative deamination of putrescine was less than 50% inhibited 1 hr after drug administration and had returned to normal by 17 hr, the next time point studied. This can be contrasted to the results obtained with isometamidium, where at 41 hr after a dose of 20 mg/kg the oxidation of putrescine was still inhibited by approximately 30%. MGBG and aminoguanidine were active against DAO both *in vitro* and *in vivo*, but they were inactive against HMT *in vitro*. Similar inhibition of DAO by aminoguanidine was

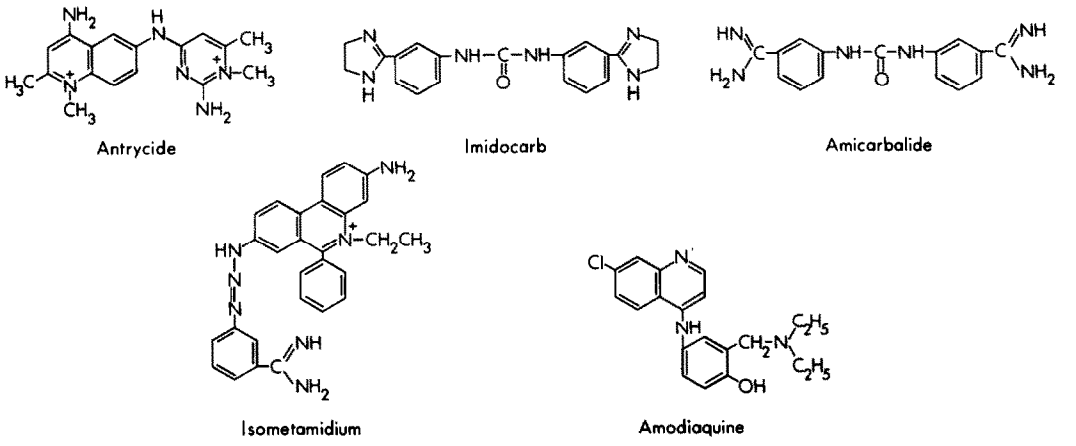

Fig. 4. Structures of inhibitors of histamine and putrescine metabolism.

obtained by Pegg and McGill [20]. In their study, $^{14}CO_2$ production after a dose of 50 mg/kg did not approach control values until 46 hr after drug administration. In this study, MGBG also inhibited putrescine oxidation *in vivo*. Production of $^{14}CO_2$ was inhibited for approximately 20 hr after a single i.p. dose of 60 mg/kg. Trimetrexate, an anticancer antifolate, shown previously [12] to be a potent inhibitor of HMT ($K_i = 7$ nM) and DAO ($K_i = 4$ μM) *in vitro*, did not inhibit putrescine oxidation *in vivo*.

Figure 4 illustrates the structures of some of the compounds used in this study. The cationic properties of the inhibitory molecules may be important in the binding of these compounds to HMT and DAO. Bacchi and coworkers [28–30] have shown an interaction between the diamidine antitrypanosomal drugs and the polyamines spermidine and spermine, both of which have putrescine as a precursor. Antrycide, ethidium and imidocarb were found to be inhibitors of the spermidine-dependent enzyme α-glycerophosphate dehydrogenase from the trypanosomatid *Leptomonas** [28]. Moreover, in the absence of spermidine, low concentrations of both Antrycide and ethidium substituted for spermidine and activated the enzyme [28]. Similar results were also obtained with pentamidine, isometamidium and imidocarb [29]. The antitrypanosomal activity of several of these drugs was also reversed by both spermidine and spermine [29–31]. Pentamidine, isometamidium, prothidium, imidocarb and amicarbalide all failed to clear the blood of parasites or prevent reappearance of the organisms when administered together with either spermidine or spermine. Blockade of drug action by polyamines does not appear to be due to interference with drug uptake, since *in vitro* incubation of blood stream form of trypanosomes with [^{3}H]pentamidine or [^{14}C]-imidocarb and either putrescine, spermidine or spermine at concentrations up to 100-fold higher than that of drug did not affect intracellular levels of the drugs* [31]. Miller and Peters [32] observed an interaction between the diamidines and polyamines in which bacteriostasis produced by propamidine was prevented by spermidine and spermine. Antrycide and imidocarb also substituted for spermine in stimulating Rausher murine leukemia virus reverse transcriptase [33].

Whether or not the effects on HA metabolism are related to antitrypanosomal activity also remains to be determined. Since neither HMT nor DAO could be detected in *T. b. brucei*, a direct effect by the drugs on the metabolism of this amine in the trypanosomes is unlikely. However, HA has been shown to be involved in resistance of cattle to the tick *Boophilus microplus* [34], in expulsion of the intestinal nematode *Nippostrongylus brasiliensis* from the rat [35], in the paralyzing action of piperazine in *Ascaris suum* [36], and on the expulsion of the intestinal nematode *Trichostrongylus colubriformis* in the guinea pig [37, 38]. Considerably more testing is required to determine whether a correlation exists between this inhibition of HA metabolism and antitrypanosomal activity *in vivo*.

In the present study only known active antitrypanosomal agents inhibited HMT and DAO *in vitro* as well as putrescine oxidation *in vivo*. Although further comparisons are needed to determine whether there are exceptions to this correlation, the evidence indicates that these biochemical tests may be useful in selecting candidate drugs for evaluation in trypanosome-infected animals. Apparently compounds which can bind to the enzymes metabolizing putrescine and HA have structural features relevant to inhibition of polyamine metabolism. Since the antitrypanosomal activity of a number of the known active compounds can be prevented by the administration of spermidine or spermine to infected mice, it is likely that polyamine-dependent metabolism in these parasitic organisms may afford a basis for the selective toxicity essential for chemotherapy.

* C. J. Bacchi, unpublished data.

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