ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 1974, p. 372-374

Vol. 6, No. 3
Printed in U.S.A

In Vitro Uptake of Isometamidium and Diminazene by *Trypanosoma brucei*

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Received for publication 18 March 1974

The cattle trypanocide, isometamidium, was readily taken up by inactivated *Trypanosoma brucei*, while its uptake by living parasites was reduced or inhibited by plasma. In both respects isometamidium differs from diminazene.

Two criteria on which Hawking and Sen (3) based their classification of trypanocides were the degree of drug uptake by the parasite under study and the effect of the drug on infectivity. They found the diamidines to be well taken up but with little effect on infectivity, whereas the inappreciably absorbed phenanthridines readily abolished infectivity. In the present study, two of the newer cattle drugs, diminazene aceturate (Berenil), a diamidine (Fig. 1), and isometamidium hydrochloride (Samorin), a phenanthridinium compound also containing an amidino group (Fig. 2), were chosen for comparative studies.

The strain of *Trypanosoma brucei* employed was isolated from an ox, adapted to rats, and maintained by serial passage. It was highly susceptible to both drugs when tested in rats and mice. Rats with peak parasitaemia were lightly anaesthetized, and blood removed by cardiac puncture was diluted 1:4 with ice-cold buffered medium of pH 8.0 (5) containing 20 IU of heparin per ml. The trypanosomes were separated by differential centrifugation (8) or by an anion exchange method (5), rapidly suspended in ice-cold suspending medium (see table footnotes), and diluted to give EEL nephelometer readings between 30 and 100, using a Unigalvo type 20. Portions of the suspensions were exposed immediately to equal volumes of drug solution in the same medium (4 to 50 μg/ml) and placed in a water bath in the dark at 37 C for 30 min. Drug controls and control suspensions were treated simultaneously in the same way. Hemocytometer counts were meanwhile made on the parasite suspension.

After incubation, the mixtures were centrifuged for 5 min at 3,000 rpm, portions of test and control suspensions having been removed for infectivity tests. The supernatant test and drug control solutions were assayed spectrophotometrically against the appropriate spectrophotometer blank. Drug uptake was measured from the decrease in absorption produced in a drug solution by the trypanosome suspension. Isometamidium was estimated at 379 nm, with correction for the slight bathochromic and hypochromic shifts caused by the presence of plasma. Anhydrous diminazene was measured at 370 nm (E 1%, 1 cm 682; Hoechst, personal communication). Complete spectra were determined to show that no interference with the method of assay was caused by distortion of the curve, due possibly to the leakage of cell constituents or to cell debris.

The degree of drug uptake was similar for both drugs by motile organisms suspended in Tyrode solution at levels of 7 to 10 μg/ml (Table 1). Organisms inactivated by glucose lack showed a much reduced diminazene uptake and a greatly increased isometamidium uptake. A similar pattern had been demonstrated previously with heat-inactivated trypanosomes, the uptake of the diamidine, stilbamidine, being greatly reduced (2), and that of certain phenanthridines greatly increased (6). This behavior of isometamidium with inactivated parasites, together with our observation that the somewhat higher drug levels which killed the parasites gave rise to very high uptakes, implied that the drug taken up by motile parasites was related to cellular damage. Attempts were therefore made to protect the cell by addition of phosphate buffer or plasma to the Tyrode solution.

The addition of phosphate buffer increased the uptake of both drugs by motile parasites to the same degree (see Tables 1 and 2). Cellular integrity was not appreciably impaired in the unbuffered medium. The addition of plasma, however, completely abolished the uptake of

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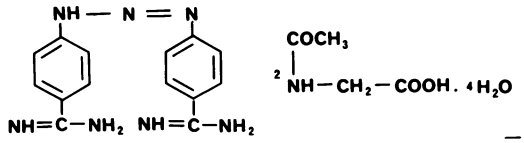

FIG. 1. *Diminazene aceturate (4,4'-diamidinodiazoaminobenzene diaceturate tetrahydrate).*

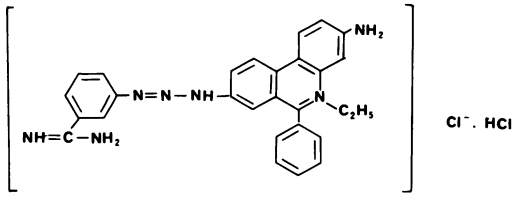

FIG. 2. *Isometamidium hydrochloride (8-m-amidinophenyldiazoamino-3-amino-5-ethyl-6-phenylphenanthridinium chloride hydrochloride).*

TABLE 2. *Relative uptakes of isometamidium and diminazene by T. brucei[a] from phosphate-buffered Tyrode solution[b] at 37 C*

Drug	Drug concn available (μg/ml)	Residual drug concn (μg/ml)	Mean drug uptake	
			μg/ml	ng/10⁶
Isometamidium	8.59 8.76	2.02 1.80	6.77	215
Diminazene (anhydrous)	10.16 10.10	1.77 1.72	8.38	266

[a] Trypanosomes were separated by anion exchange. Trypanosome density, 31.5×10^6 per ml.

[b] Equal parts by volume of 0.1 M phosphate buffer at pH 7.3 and a modified Tyrode solution (NaCl, 0.8%; KCl, 0.02%; $CaCl_2$, 0.02%; $MgCl_2 \cdot 6H_2O$, 0.02%, and glucose, 0.8%).

isometamidium except at very high levels, whereas it had no effect on diminazene uptake (Table 3). This difference may be explained on the basis that isometamidium is known to bind readily with serum albumin (7), but no binding has been reported with diminazene. Binding may thus reduce the availability of isometamidium so that none is available for uptake at the lower levels, whereas at the high level there may be adequate unbound drug to give a degree of uptake commensurate with cellular damage. The mechanisms of uptake of the two drugs by living trypanosomal cells are therefore clearly different. The uptake of diminazene indicates the involvement of some active process which is not affected by plasma or drug concentration, and that of isometamidium is severely restricted unless there is cellular damage.

The blood concentration attained in vivo after administration of curative doses appears to differ considerably; the peak concentration for diminazene has been recorded as 2 to 7 μg/ml (1), but that of isometamidium may well be lower than 0.2 μg/ml (4). Under these circumstances, the amount of isometamidium taken up would be very small in comparison with diminazene, in which the uptake is probably similar to what it is in vitro. If drug uptake is a prerequisite to trypanocidal action, we can conclude that isometamidium is an extremely potent drug.

Both drugs were found to abolish infectivity at the lowest concentrations employed (approximately 5 μg/ml).

TABLE 1. *Relative uptakes of isometamidium and diminazene by motile and inactivated T. brucei from Tyrode solution[a] at 37 C[b]*

Drug	Drug concn available (μg/ml)	Residual drug concn (μg/ml)		Mean drug uptake			
				μg/ml		ng/10⁶	
		Motile	Inactivated	Motile	Inactivated	Motile	Inactivated
Isometamidium	7.01 7.21	4.72 4.88	2.08 1.92	2.31	5.11	112	247
Diminazene (anhydrous)	9.22 9.09	6.41 6.44	8.80 8.80	2.73	0.36	132	17

[a] NaCl, 0.8%; KCl, 0.02%; $CaCl_2$, 0.02%; $MgCl_2 \cdot 6H_2O$, 0.02%; $NaHCO_3$, 0.02%; NaH_2PO_4, 0.01%, and glucose, 0.4%. The pH of the solution was 7.3.

[b] Glucose-free Tyrode solution was used to suspend one fraction of the dense parasite suspension, and served to immobilize the parasites after 15 min at room temperature. Drug solutions were prepared in the same medium. Trypanosome density, 20.7×10^6 per ml. Trypanosomes were separated by centrifugation.

TABLE 3. *Effect of drug concentration and added plasma on uptakes of isometamidium and diminazene by T. brucei*[a] *from Tyrode solution*[b] *at 37 C*

Drug	No. of trypanosomes/ml	Drug concn available (μg/ml)	Residual drug concn (μg/ml)	Mean drug uptake	
				μg/ml	ng/10⁶
Isometamidium	12.0×10^6	39.7	34.0	6.1	508
		42.8	36.4		
		8.63	9.21	−0.37	
		8.85	9.01		
		4.87	4.90	−0.09	
		4.79	4.94		
Diminazene (anhydrous)	21.0×10^6	46.1	42.2	3.3	160
		45.5	42.8		
		9.16	6.23	2.93	140
		9.12	6.19		
		4.56	2.29	2.34	110
		4.68	2.26		

[a] Trypanosomes were separated by centrifugation.
[b] Inactivated human plasma was added to Tyrode solution in the proportion of 1:3 (vol/vol).

We thank May and Baker Ltd., Dagenham, and Farbwerke Hoechst, Frankfurt (Main), respectively, for their generous supplies of Samorin and Berenil.

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