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In Vitro and In Vivo Activities of Trybizine Hydrochloride against Various Pathogenic Trypanosome Species

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Trybizine hydrochloride [*O*,*O*′-bis(4,6-diamino-1,2-dihydro-2,2-tetramethylene-*s*-triazine-1-yl)-1,6-hexanediol dihydrochloride] was active in vitro against the sleeping sickness-causing agents *Trypanosoma brucei* subsp. *rhodesiense* and *T. brucei* subsp. *gambiense*; against a multidrug-resistant organism, *T. brucei* subsp. *brucei*; and against animal-pathogenic organisms *Trypanosoma evansi*, *Trypanosoma equiperdum*, and *Trypanosoma congolense*; but not against the intracellular parasites *Trypanosoma cruzi* and *Leishmania donovani*. Cytotoxic effects against mammalian cells were observed at approximately 10^6-fold higher concentrations than those necessary to inhibit *T. brucei* subsp. *rhodesiense*. Trybizine hydrochloride was able to eliminate *T. brucei* subsp. *rhodesiense* and *T. brucei* subsp. *gambiense* in an acute rodent model with four intraperitoneal doses of 0.25 mg kg of body weight^{-1} or four doses of 1 mg kg^{-1}, respectively, or with four oral doses of 20 mg kg^{-1}. The compound expressed activity against suramin-resistant *T. evansi* strains in mice. However, these concentrations were not sufficient to cure mice infected with multidrug-resistant *T. brucei* subsp. *brucei*. A late-stage rodent model with central nervous system involvement could not be cured, indicating that trybizine may not pass the blood-brain barrier in sufficient quantities.

Current methods of treatment of African sleeping sickness are unsatisfactory because the number of available drugs is limited, the period of treatment is long, and the treatment is associated with severe side effects. Melarsoprol (Arsobal; Specia, Paris, France) has adverse effects (17), while the only alternative drug for the late-stage disease, DL-α-difluoromethylornithine (DFMO; Eflornithine), is only effective against gambiense sleeping sickness but not against the rhodesiense type (1, 7, 8). In addition, the occurrence of drug-resistant trypanosomes is threatening successful chemotherapy of human trypanosomosis (15a) as well as animal trypanosomoses (2). For Chagas disease and the leishmaniases, the existing drugs are also inadequate because of their variable efficacy, toxicity, and required long courses of treatment (3).

A novel antitrypanosomal agent has been introduced by the Shanghai Institute of Pharmaceutical Industry. Trybizine hydrochloride [*O*,*O*′-bis(4,6-diamino-1,2-dihydro-2,2-tetramethylene-*s*-triazine-1-yl)-1,6-hexanediol dihydrochloride; Chinese patent, CN 1096514A] has been shown to express activity against *Trypanosoma evansi*, a trypanosome species infecting various domestic animals worldwide. The aim of this study was to evaluate trybizine hydrochloride for its activity against other pathogenic hemoflagellates, particularly those which cause human sleeping sickness (*Trypanosoma brucei* subsp. *rhodesiense* and *T. brucei* subsp. *gambiense*), Chagas disease (*Trypanosoma cruzi*), and leishmaniasis (*Leishmania donovani*).

MATERIALS AND METHODS

Parasites and cells. The history of the trypanosome stocks and clones used in this study is given in Table 1. The culture-adapted populations of *T. brucei* subsp. *brucei* STIB 950 and STIB 940 show a multidrug-resistant phenotype (10, 11). All Sudanese *T. evansi* strains used in this study were resistant in vitro and in mice to quinapyramine and suramin (6). *T. evansi* STIB 780 is highly resistant to quinapyramine and suramin (22). *T. evansi* STIB 806 is resistant to isometamidium, and *T. evansi* STIB 780 is resistant to quinapyramine and suramin. Both *T. congolense* STIB 801 and STIB 790 are resistant to diminazene and isometamidium.

L. donovani MHOM/ET/67/L82 and *T. cruzi* MHOM/Br/00/Y were propagated in mouse peritoneal macrophages and in the human fetal lung fibroblast cell line WI-38 (ATCC CCL 75), respectively. In addition, rat skeletal muscle myoblast (L-6) cells and human adenocarcinoma (HT-29) cells, isolated in 1964 from a primary tumor (ATCC HTB 38), were used.

Drugs. Trybizine hydrochloride (Fig. 1) was obtained from W. Zhou from the Shanghai Institute of Pharmaceutical Industry. The compound was solubilized in dest. H_2O before use at 1 mg of drug/ml.

Cultivation of parasites. *T. brucei* subsp. *rhodesiense*, *T. brucei* subsp. *gambiense*, *T. brucei* subsp. *brucei*, *T. evansi*, and *Trypanosoma equiperdum* were propagated in vitro in minimum essential medium (MEM; GIBCO-BRL no. 072-1100 powder) with Earle's salts supplemented with 1 mg of glucose ml^{-1}, 1% MEM nonessential amino acids (100×), 2.2 mg of $NaHCO_3$ ml^{-1}, and 10 mM HEPES. The medium was further supplemented with 2 mM sodium pyruvate, 0.2 mM 2-mercaptoethanol, 0.1 mM hypoxanthine, and 15% heat-inactivated horse serum (prepared by us from horse blood obtained from a local slaughterhouse). The medium for *T. brucei* subsp. *gambiense* cultures was supplemented with 10% human serum (STI human serum pool) and 5% fetal bovine serum (Biological Industries, Kibbutz Beth Haemek, Israel), both heat inactivated. *T. congolense* isolates were propagated according to the method of Kaminsky et al. (14) in Iscove's medium (GIBCO-BRL no. 074-02200; Life Technologies, Basel, Switzerland) supplemented with 0.05 mM bathocuproinedisulfonic acid, 1.5 mM L-cysteine, 0.5 mM hypoxanthine, 2 mM L-glutamine, 0.12 mM 2-mercaptoethanol, 2 mM sodium pyruvate, and 15% heat-inactivated goat serum (C.C.PRO GmbH, Karlsruhe, Germany).

All cultures were kept in 24-well plates (Costar, Cambridge, Mass.) at 37°C (or 34°C for *T. congolense*) in a humidified atmosphere in 5% CO_2. Cultures were subpassaged to a density of 10^3 to 10^5 trypanosomes per ml every second or third day. Trypanosomes in the logarithmic growth phase were used for determination of drug sensitivities.

The medium for cultivation of *T. cruzi* consisted of MEM (GIBCO-BRL no. 072-1100 powder) supplemented with 1% MEM nonessential amino acids

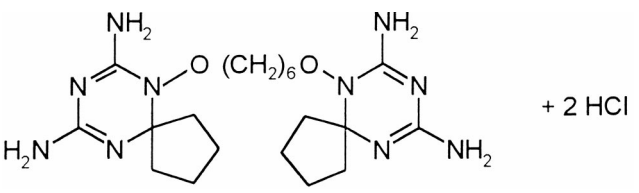

FIG. 1. Chemical structure of trybizine hydrochloride.

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TABLE 1. History of the trypanosome stocks used in this study

Designation of derivative	Species	Original designation	Place of isolation	Yr of isolation	Host
STIB 900	*T. brucei* subsp. *rhodesiense*	STIB 704	Tanzania	1982	Human
STIB 930	*T. brucei* subsp. *gambiense*	TH-1/78E(031)	Ivory Coast	1978	Human
STIB 920	*T. brucei* subsp. *brucei*	STIB 348	Tanzania	1971	Hartebeest
STIB 950	*T. brucei* subsp. *brucei*	CP 2469	Somalia	1985	Bovine
STIB 940	*T. brucei* subsp. *brucei*	CP 547	Somalia	1985	Bovine
GVR 35	*T. brucei* subsp. *brucei*	LUMP 22	Tanzania	1966	Wildebeest
STIB 806	*T. evansi*		China	1983	Buffalo
EASTRY 1	*T. evansi*		Sudan	1994	Camel
EASTRY2	*T. evansi*		Sudan	1994	Camel
WESTRY3	*T. evansi*		Sudan	1996	Camel
WESTRY4	*T. evansi*		Sudan	1996	Camel
STIB 780	*T. evansi*	CP 893	Kenya	1982	Camel
STIB 818	*T. equiperdum*		China	1979	Horse
STIB 910	*T. congolense*	STIB 249	Tanzania	1971	Lion
CP 81	*T. congolense*		Kenya	1966	Bovine
STIB 801	*T. congolense*	IL 2856	Burkina Faso	1983	Bovine
STIB 790	*T. congolense*	CP 2036	Kenya	1985	Bovine

(100×) and 10% heat-inactivated fetal bovine serum. Monolayers of WI-38 or L-6 cells were subsequently infected with trypomastigote forms of *T. cruzi*.

All mammalian cells were propagated in MEM supplemented with 10% heat-inactivated fetal bovine serum. Stock cultures of mammalian cells were maintained in T-25 flasks (Falcon, Becton Dickinson) in a humidified atmosphere at 37°C in 5% CO_2. Cells were subpassaged to the appropriate split ratio (1:4 to 1:6) once a week.

In vitro chemosensitivity assays. Drug susceptibilities were determined in vitro as previously described (18, 19). In vitro activity of trybizine hydrochloride against *T. cruzi* was determined with a 5-day assay developed in our laboratory (unpublished). WI-38 cells were seeded in a density of 10^5 cells ml^{-1} in 1-ml samples into 24-well culture plates (Costar). After 48 h, the medium was removed, and the cell layer was infected with 10^5 trypomastigote *T. cruzi* organisms. The infection was allowed to develop for 48 h, after which the medium was replaced with fresh medium containing the appropriate drug concentration. Propagation of amastigotes and the appearance of trypomastigotes under drug pressure were determined microscopically after an additional 72-h exposure period. The susceptibility of *L. donovani* to trybizine hydrochloride in vitro was tested by the procedure described by Neal and Croft (16).

In vivo drug susceptibility test. Female Swiss ICR mice, weighing 25 to 35 g each, were used for the in vivo drug tests. Each mouse was inoculated intraperitoneally (i.p.) with 10^5 trypanosomes, and treatment was initiated 24 h after inoculation. Trybizine hydrochloride was administered i.p. or orally at the appropriate concentration. The tail blood of mice was examined for the presence of trypanosomes three times a week for a total of 60 days by the wet blood film technique. Mice were considered cured when no trypanosomes were detected during the observation period. A similar procedure was used to evaluate the activity of trybizine hydrochloride against *T. brucei* subsp. *gambiense*, except that *Mastomys natalensis* rats were used instead of white mice. *M. natalensis* were immunosuppressed prior to infection with 200 mg of cyclophosphamide kg of body $weight^{-1}$. The tail blood of *Mastomys* was examined for the presence of trypanosomes by the hematocrit centrifugation technique (21).

To evaluate the activity of trybizine hydrochloride against central nervous system (CNS) infections, the rodent late-stage model according to Jennings and Gray (9) was used.

Time-versus-dose experiment. Experiments to determine the time of exposure to a drug versus the viability (time-dose response) of *T. brucei* subsp. *brucei* STIB 920 in the presence of trybizine hydrochloride were performed as previously described (12).

RESULTS

The effects of the in vitro activity of trybizine hydrochloride on various hemoflagellates and on mammalian cells are summarized in Table 2. Trybizine eliminated all *T. brucei* subsp. *rhodesiense* and *T. brucei* subsp. *gambiense* organisms at a concentration of or below 1.3 ng ml^{-1}. The multidrug-resistant *T. brucei* subsp. *brucei* stocks were less susceptible, and the difference in susceptibility between the susceptible and multi-

TABLE 2. In vitro activity of trybizine hydrochloride against various trypanosome species, *L. donovani*, *T. cruzi*, and mammalian cells

Species	Trypanosome stock	Result (ng ml^{-1}) for [a]:			
		Trybizine hydrochloride		Diminazene aceturate	
		MIC	IC_{50}	MIC	IC_{50}
T. brucei subsp. *rhodesiense*	STIB 900	0.4 ± 0	0.04 ± 0.02	22.6 ± 10.3	5.22 ± 0.60
T. brucei subsp. *gambiense*	STIB 930	2.7 ± 1.1	0.20 ± 0.51	48.4 ± 45.8	ND[b]
T. brucei subsp. *brucei* mdr	STIB 940	28.0 ± 10.5	5.84 ± 2.09	193.0 ± 121.2	56.0 ± 9.80
T. brucei subsp. *brucei* mdr	STIB 950	28.5 ± 10.4	7.20 ± 7.76	185.0 ± 128.1	28.0 ± 2.98
T. brucei subsp. *brucei*	STIB 920	2.6 ± 1.2	0.84 ± 0.53	30.8 ± 7.7	5.39 ± 0.99
T. evansi	STIB 806	0.2 ± 0.1	0.06 ± 0.04	ND	ND
T. equiperdum	STIB 818	0.1 ± 0	0.04 ± 0.02	ND	ND
T. congolense	STIB 910	11.1 ± 0	2.21 ± 0.43	111 ± 0	63.2 ± 9.03
T. congolense	CP 81	11.1 ± 0	2.19 ± 0.09	111 ± 0	64.5 ± 9.70
L. donovani	MHOM/ET/67/L82	$>9 \times 10^4$	NA[c]	NA	NA
T. cruzi	MHOM/Br/00/Y	$>1 \times 10^5$	NA	NA	NA
Mouse L-6 cells		1×10^6	NA	NA	NA
Human HT-29 cells		$>1 \times 10^6$	NA	NA	NA

[a] MICs and IC_{50}s are given as means ± standard deviations of at least three to five experiments, each performed in duplicate. For details, see Materials and Methods.
[b] ND, not done.
[c] NA, not applicable.

TABLE 3. Time-dose response of *T. brucei* subsp. *brucei* STIB 920 to trybizine hydrochloride in vitro[a]

Trybizine hydrochloride concn (μg ml^{-1})	Result with drug exposure time (h)[b]:										
	0.5	1	3	6	8	16	24	48	72	96	144
100	−	−	−	−							
10		+	+	+	+/−	−	−				
1		+	+	+	+	+	+/−	−			
0.1		+	+	+	+	+	+	−			
0.01						+	+/−	−	−	−	−
0.001							+	+	+	+	+
0.0001									+	+	+

[a] Trypanosome cultures were observed daily for 10 days following the indicated drug exposure time.

[b] +, trypanosomes were not affected by the drug; +/−, drug-induced growth inhibition of trypanosomes (In some cases, cultures recovered to normal growth.); −, drug-induced elimination of trypanosomes.

drug-resistant *T. brucei* subsp. *brucei* organisms was 10-fold. *T. evansi* and *T. equiperdum* were very susceptible to trybizine; the MICs (0.2 and 0.1 ng ml^{-1}) for them were the lowest obtained for all trypanosome species. *T. congolense*, a cattle-pathogenic species, was 50- to 100-fold less-susceptible to trybizine. Overall, the MIC and the 50% inhibitory concentration (IC_{50}) for the most and least susceptible stocks differed 280-fold.

No activity was observed against the intracellular *T. cruzi* and *L. donovani* at the highest concentrations tested. Mouse L-6 cells were only affected at a concentration of 1 mg ml^{-1}. This concentration did not affect human HT-29 epithelial cells.

Investigations of the time-dose response of trybizine in *T. brucei* subsp. *brucei* STIB 920 revealed that an exposure of 10 μg ml^{-1} over 16 h was necessary to eliminate all trypanosomes. When the exposure time was extended to 48 h, a concentration of 10 ng ml^{-1} was sufficient; the same effect was achieved with 1 and 0.1 μg ml^{-1} over 48 h. It was not possible to inhibit *T. brucei* subsp. *brucei* irreversibly with a concentration of or below 1 ng ml^{-1} (Table 3).

Trybizine and suramin had an antagonistic effect on *T. brucei* subsp. *brucei* STIB 920, as demonstrated by the isobologram of fractional IC_{50}s (Fig. 2A). The same antagonistic effect was observed when trybizine was used in combination with diminazene aceturate (Fig. 2B). An additive effect was observed for the combination of trybizine with quinapyramine (Fig. 2C).

The results for the activity of trybizine hydrochloride in infected rodents are summarized in Table 4. It was possible to cure mice infected with human-pathogenic *T. brucei* subsp. *rhodesiense* when trybizine hydrochloride was applied i.p. at four doses of 0.25 mg kg^{-1}. *T. brucei* subsp. *gambiense*-infected rodents were cured with four doses of 1 mg kg^{-1}. Importantly, cure was achieved when trybizine was applied orally with four doses of 20 mg kg^{-1}. However, it was not possible to cure mice infected with multidrug-resistant *T. brucei* subsp. *brucei*. Neither was it possible to cure the late-stage CNS model of mice infected with *T. brucei* subsp. *brucei* GVR 35. The result was the same even after combination treatment of trybizine hydrochloride with DFMO or suramin.

Some of the equine-pathogenic *T. evansi* strains including isolates resistant to quinapyramine and suramin were eliminated with four doses of 1 mg kg^{-1}. However, three *T. evansi* strains could not be cured in mice at all. Only one of three tested cattle-pathogenic *T. congolense* strains was eliminated with four doses of 1 mg kg^{-1}. For the others, four doses of 2.5 mg kg^{-1} were not sufficient to achieve a cure in mice.

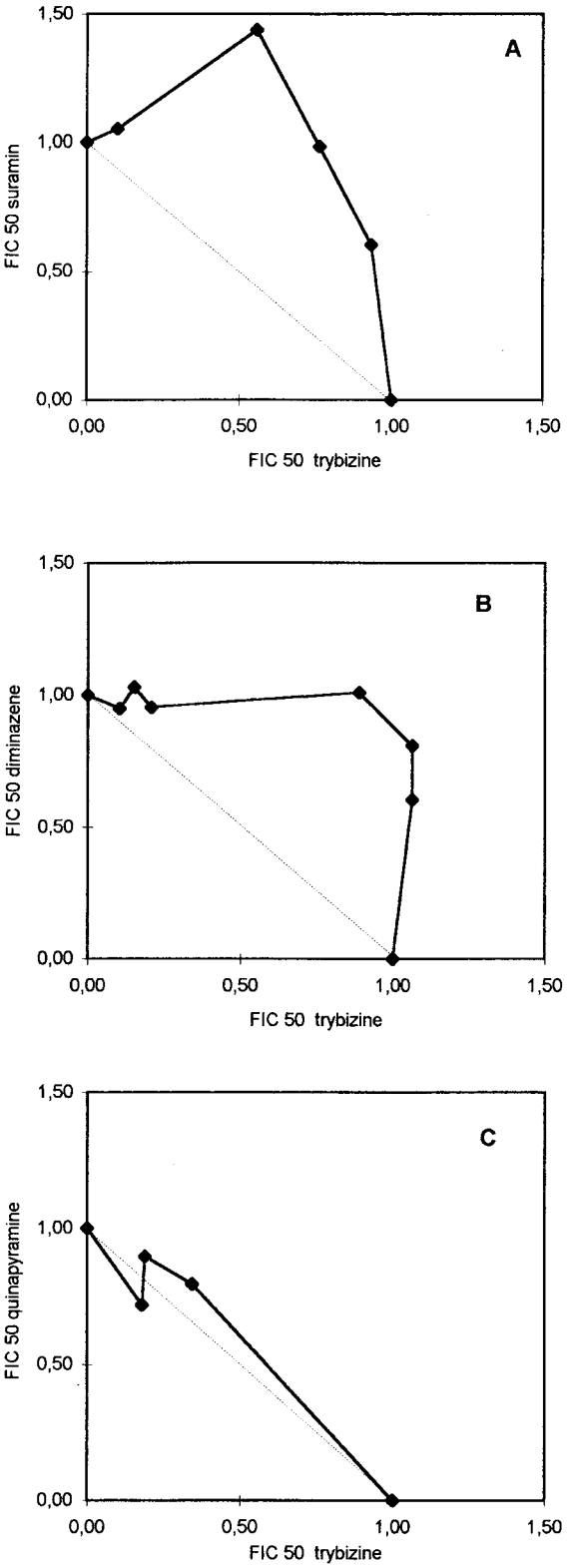

FIG. 2. Isobolograms of trybizine and the current trypanocides suramin, diminazene, and quinapyramine. Control IC_{50}, normalized to 1 U of the IC_{50}, refers to trybizine alone (X-axis [FIC 50, fractional IC_{50}]) and to suramin (A), diminazene (B), and quinapyramine (C). The solid line represents the isobole of the drug combination in vitro. The dotted line joining the FICs of 1 is the isobole of an additive combination (C). A convex isobole represents an antagonistic combination (A and B).

TABLE 4. Antitrypanosomal activity of trybizine hydrochloride against various trypanosome species in rodent models

Species	Stock	Drug susceptibility	Disease model	Dose in mg kg^{-1} (no. of doses)	Route	No. of rodents cured/ no. treated
T. brucei subsp. *rhodesiense*	STIB 900	Susceptible	Acute	0.1 (4)	i.p.	2/4
				0.25 (4)	i.p.	4/4
				1 (1)	i.p.	3/4
				5 (4)	Oral	0/4
				20 (4)	Oral	4/4
T. brucei subsp. *gambiense*	STIB 930	Susceptible	Acute	0.5 (5)	i.p.	3/4
				1 (4)	i.p.	4/4
T. brucei subsp. *brucei*	STIB 950	Multidrug resistant (including diminazene)	Acute	0.25 (4)	i.p.	0/4
				1 (4)	i.p.	0/3
				2.5 (4)	i.p.	0/4
				5 (4)	i.p.	2/4[a]
	GVR 35	Susceptible	Late stage	5 (10)	i.p.	0/4
				2.5 (7)	i.p.	0/2
				4 (14) (DFMO)[b]	Oral	
				2.5 (5)	i.p.	0/2
				10 (5) (suramin)[b]	i.p.	
T. evansi	Eastry 1	Resistant to suramin and quinapyramine	Acute	1 (4)	i.p.	0/4
	Eastry 2	Resistant to suramin and quinapyramine	Acute	1 (4)	i.p.	7/8
	Westry 3	Resistant to suramin and quinapyramine	Acute	1 (4)	i.p.	4/4
	Westry 4	Resistant to suramin and quinapyramine	Acute	2.5 (4)	i.p.	0/4
	STIB 806	Resistant to isometamidium	Acute	1 (4)	i.p.	4/4
	STIB 780	Resistant to suramin and quinapyramine	Acute	2.5 (4)	i.p.	0/4
T. congolense	STIB 801	Resistant to diminazene and isometamidium	Acute	1 (4)	i.p.	0/4
	CP 81	Susceptible	Acute	2.5 (4)	i.p.	0/4
	STIB 790	Resistant to diminazene and isometamidium	Acute	1 (4)	i.p.	4/4
	STIB 910	Susceptible	Acute	2.5 (4)	i.p.	0/4

[a] Two mice died during treatment because of toxicity.
[b] Trybizine hydrochloride-DFMO or -suramin combination.

DISCUSSION

The results obtained clearly demonstrate that trybizine hydrochloride is a powerful antitrypanosomal compound with a specific activity in vitro comparable to melarsoprol (14). Importantly, the cytotoxicity for mammalian cells was very low if at all detectable, which made trybizine a candidate for in vivo evaluation (14).

In mice, trybizine hydrochloride was able to eliminate both human-pathogenic trypanosome subspecies after either i.p. or oral administration. The latter is particularly important, because all currently available trypanocides against human trypanosomiasis have to be applied parenterally or intravenously (15), with the exception of DFMO, which can also be given orally (5). Furthermore, most of the mice infected with quinapyramine- and suramin-resistant *T. evansi* strains were cured. Thus, our in vitro and in vivo results confirm the activity of trybizine hydrochloride observed against Chinese *T. evansi* in buffaloes and bovines (21a). Trybizine has great potential against *T. evansi*, because quinapyramine and suramin resistance appears to be a serious problem in the chemotherapy of surra (6, 22) and, therefore, may become an alternative drug to Cymelarsan. The first trials with the arsenical agent Cymelarsan against *T. evansi* were carried out by Tager-Kagan et al. (20). However, it has been shown that there is some cross-resistance of Cymelarsan to other trypanocides (22).

The compound showed reduced activity for multidrug-resistant *T. brucei* subsp. *brucei* and for *T. congolense*. This reduced in vitro sensitivity is reflected by the in vivo results. The multidrug-resistant strain *T. brucei* subsp. *brucei* STIB 950 could not be cured and neither could three of the four *T. congolense* strains tested. The mechanisms for the resistance of the *T. brucei* subsp. *brucei* strains are not known, since the mode of action of trybizine is not known yet. The nonresponsiveness of both multidrug-resistant *T. brucei* subsp. *brucei* and *T. congolense* is a serious drawback for the potential development of trybizine for treatment of tsetse fly-transmitted trypanosomoses in sub-Saharan Africa, because drug resistance is a major problem in chemotherapy of livestock trypanosomosis, and

T. congolense is a major cattle-pathogenic species (2). Experiments with domestic animals are needed to confirm the nonresponsiveness of *T. congolense*.

A crucial issue for assessment of the potential of any new compound against human trypanosomosis is the ability of such a compound to cross the blood-brain barrier, because in the progress of the disease, trypanosomes invade the CNS. So far, of all current trypanocides, only melarsoprol and DFMO are able to cross the blood-brain barrier in sufficient quantities (4). Trybizine hydrochloride was not able to cure the late-stage CNS model (Table 4) in mice, which would indicate that trybizine is unable to build up therapeutic levels in the CNS. Unambiguous evidence may be given by exploratory pharmacokinetic experiments with monkeys, which are in progress.

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