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RESEARCH COMMUNICATION

Evaluation of a short-term *in vitro* growth-inhibition test to determine susceptibility of *Trypanosoma vivax* stocks to various trypanocides

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ABSTRACT

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Two *Trypanosoma vivax* stocks were initiated in culture with tsetse or culture-derived metacyclics. They were propagated axenically as bloodstream trypomastigotes at 35 °C in 4 % CO_2 in air. Populations of trypanosomes were incubated with various concentrations of antitrypanosomal compounds, namely diminazene aceturate, quinapyramine sulphate, DL-α-difluoromethylornithine, isometamidium chloride, suramin and mel Cy. Growth was monitored after 24 h of incubation and the growth inhibition was calculated. All six drugs tested showed little effect upon the growth of the parasite populations. These results indicate that a 24-h growth-inhibition test was not suitable for determining the drug susceptibility of *T. vivax* stocks *in vitro*. Neither did the results correlate with those obtained with susceptible or resistant stocks of *T. b. brucei*, *T. b. evansi* or *T. simiae* described in the literature, or with the results of these two *T. vivax* stocks tested in cattle.

A long-term *in vitro* viability assay was recently used to examine *Trypanosoma vivax* stocks for their susceptibility to isometamidium chloride (Zweygarth, Kaminsky & Gray 1991b). These experiments showed that isometamidium-sensitive and -resistant *T. vivax* stocks could be distinguished *in vitro*. In the present study, we used parasite populations derived from a continuous axenic culture system of *T. vivax* (Zweygarth, Gray & Kaminsky 1991a), to determine their susceptibility to various antitrypanosomal drugs in a short-term (24 h) growth-inhibition test (Kaminsky & Zweygarth 1989).

Trypanosoma vivax stock CP 2171 (originally designated as TV 5) was isolated from a Friesian cow in 1986 in Bamburi, Kenya, and stock CP 2331 (originally designated as TV 3) was isolated from a cow in 1986 in Kipini, Kenya. Both the *T. vivax* stocks have been characterized for their drug susceptibility in cattle by Schönefeld, Röttcher & Moloo (1987). The results are summarized in Table 1.

The *T. vivax* stocks were propagated in a combination of Iscove's modified Dulbecco's and RPMI 1640 medium (IMDM + RPMI 1640 XT powder medium;

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TABLE 1 Susceptibility of *Trypanosoma vivax* stocks CP 2171 and 2331 to antitrypanosomal drugs in cattle*

Drug (dosage in mg/kg)	Cured/treated cattle	
	CP 2171	CP 2331
Diminazene (3,5)	11/12	3/3
Isometamidium (2)	0/3	0/3
Quinapyramine sulphate (4,4)	0/3	3/3

* Schönefeld *et al*. 1987

TABLE 2 Susceptibility of *Trypanosoma vivax* stocks CP 2171 and 2331 to antitrypanosomal drugs *in vitro*

Drugs	IC_{50} (μg/mℓ)	
	CP 2171	CP 2331
Diminazene aceturate	4,34	0,43
Isometamidium chloride	4,14	1,50
Quinapyramine sulfate	0,54	0,25
Suramin	64,10	77,00
Cymelarsan	0,40	0,47
DFMO	> 100	> 100

Serva Feinbiochemica, Heidelberg, Germany) supplemented with 20 % (v/v) heat-inactivated (56 °C, 30 min) bovine serum, containing, in addition, 2 mM L-glutamine, 100 IU/mℓ penicillin, 100 μg/mℓ streptomycin, 0,2 mM adenosine, 0,1 mM 2-mercaptoethanol (2-ME) (Baltz, Baltz, Giroud, Crockett 1985), 1 mM L-cysteine and 0,02 mM 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline disulfonic acid (bathocuproine sulfonate, BCS; Serva Feinbiochemica) (Yabu, Takayanagi & Sato 1989).

To initiate bloodstream-form cultures of *T. vivax* stock CP 2171, tsetse-fly-derived metacyclics were obtained from infected *Glossina morsitans centralis*, by placing the infected hypopharynges into a culture well so that the metacyclics moved out into the medium. The culture-derived metacyclics (Kaminsky, Chuma, Zweygarth, Kitosi & Moloo 1991) of stock CP 2331 were used to initiate bloodstream-form cultures. Metacyclics were separated from epimastigotes by the method of Hirumi, Nelson, Hirumi, Moloo & Nantulya (1985). Supernatants from insect-form cultures were incubated with 10 % (v/v) bovine plasma at 27 °C for 1 h, which caused agglutination of the non-infective culture forms. Metacyclics were then separated by passage through a 5-μm pore filter. Propagation of bloodstream-form cultures of *T. vivax* has been described previously (Zweygarth *et al*. 1991a).

Diminazene aceturate (Berenil, Hoechst AG, Frankfurt, Germany), isometamidium chloride (Samorin, May & Baker, Dagenham, U.K.), quinapyramine sulphate (Trypacide, May & Baker, Dagenham, U.K.), and suramin (Naganol, Bayer AG, Leverkusen, Germany) were purchased commercially. The following compounds were kindly donated by the following drug companies: Mel Cy (Cymelarsan, Rhône Mérieux, Toulouse, France), and DL-α-difluoromethylornithine hydrochloride monohydrate (DFMO; eflornithine; Ornidyl; Merrell Dow, Cincinnati, Ohio, USA).

The growth-inhibition test was carried out as described by Kaminsky & Zweygarth (1989) for *T. b. brucei* and *T. b. evansi*. Suspensions of trypanosomes derived from axenic cultures were adjusted to a concentration of 2×10^5 mℓ, and 375 μℓ were pipetted into each well of a 48-well culture plate (Costar, Cambridge, Mass., USA). An equal volume of 2x concentrated drug solution in medium was added. Each drug concentration was tested in duplicate, and was repeated at least twice. After 24 h of incubation, 500 μℓ aliquots were removed from each well, transferred into disposable cups (Sarstedt, Nümbrecht, Germany), fixed with 6 μℓ formalin (37 %), diluted with Isoton (Coulter Electronics, Nairobi, Kenya) and counted in a Coulter Counter model ZM (70 μm aperture). The number of generations in drug-treated cultures was calculated for each well and the relative growth of trypanosome populations was determined by comparison with the number of generations (100 %) in control cultures.

The results of the drug-susceptibility tests carried out for both T. vivax stocks are summarized in Table 2. Animals infected with either stock were cured with the recommended dose of 3,5 mg/kg diminazene (Schönefeld *et al*. 1987). However, the IC_{50} values of 0,43 and 4,34 μg/mℓ, respectively, are out of the range for those found for other diminazene-susceptible trypanosome species tested with the same system. *Trypanosoma b. brucei*, *T. b. evansi* (Kaminsky & Zweygarth 1989) and *T. simiae* (Zweygarth, Moloo & Kaminsky 1993) had IC_{50} values approximately ten times lower than *T. vivax* stock CP 2331. The antitrypanosomal action of diminazene is believed to be partially due to the inhibition of the kinetoplast DNA synthesis (Newton & LePage 1967). Diminazene also inhibited S-adenosyl-L-methionine (AdoMet) decarboxylase in *T. b. brucei*, an enzyme for the biosynthesis of polyamines, and its inhibition might contribute to the overall efficacy of diminazene as a trypanocide (Bitonti, Dumont & McCann 1986). The combined action of diminazene on both targets within the trypanosome would probably be more deleterious than the single action on either of the two targets. Therefore it is possible that the AdoMet decarboxylase of *T. vivax* is inhibited to a lesser extent than that of *T. b. brucei* or *T. simiae*. This would explain the great discrepancy of the *in vitro* results between *T. vivax* and the other trypanosome species examined. *In vivo*, however, the action on DNA synthesis would be sufficient because of the support of the host's immune system to clear the parasites from the circulation.

The average IC_{50} values of isometamidium for *T. vivax* in the present experiments, i.e. 4,14 μg/mℓ for stock

CP 2171 and 1,5 μg/ml for stock CP 2331, indicate a high level of drug resistance *in vitro*. However, comparing previous data from a long-term viability assay obtained with stock CP 2331 (Zweygarth *et al.* 1991b), we found that they were almost identical with results obtained for a stock of *T. simiae* (CP 813) (Zweygarth *et al.* 1993). Surprisingly, with the short-term growth-inhibition assay on the IC_{50} basis, the *T. vivax* stock was almost ten times more resistant than *T. simiae*.

Similarly, for quinapyramine sulphate the IC_{50} values were more than 160 times higher than those found for *T. simiae* stocks (Zweygarth *et al.* 1993) although one of the *T. vivax* stocks (CP 2331) was eliminated at the recommended dose of 4,4 mg/kg in cattle.

In view of the IC_{50} values obtained for a suramin-resistant *T. b. evansi* stock (22 μg/ml) (Kaminsky & Zweygarth 1989), suramin was not expected to eliminate *T. vivax* infections with their even higher IC_{50} values of 64,1 and 77 μg/ml, respectively. *Trypanosoma vivax* has therefore been considered as non-susceptible to suramin treatment, which could also be concluded from the *in vitro* results.

Mel Cy, a melaminyl-substituted phenylarsonate earmarked for the use against surra (Raynaud, Sones & Friedheim 1989), was included in the tests although mel Cy was ineffective against a mouse-infective *T. vivax* stock (unpublished results). The fact that mel Cy is not effective against *T. vivax* was confirmed *in vitro* since the IC_{50} values were more than 26 and 34 times higher than those values for a resistant *T. b. brucei* stock (Zweygarth & Kaminsky 1990).

The new antitrypanosomal compound, DFMO, which was effective in man at all stages of the Gambian sleeping sickness (Van Nieuwenhowe, Schechter, Declercq, Bone, Burke & Sjoerdsma 1985), showed only a little activity against both *T. vivax* stocks as expressed by its IC_{50} values of more than 100 μg/ml, the highest concentration used, as growth was inhibited by about 25 % only (data not shown).

In conclusion, the present experiments indicate that a short-term growth-inhibition test has its limitations in determining drug susceptibilities of *T. vivax* stocks *in vitro*. Whether this was due to the culture-test system, the peculiarity of the parasites examined, or both, needs to be elucidated. None of the results correlated with those obtained with susceptible or resistant stocks of *T. b. brucei*, *T. b. evansi*, and *T. simiae in vitro*, nor with those of the *T. vivax* stocks used in cattle.

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REFERENCES

BALTZ, T., BALTZ, D., GIROUD, C. & CROCKETT, J. 1985. Cultivation in a semi-defined medium of animal-infective forms of *Trypanosoma brucei, T. equiperdum, T. evansi, T. rhodesiense* and *T. gambiense*. *EMBO Journal*, 4:1273–1277.

BITONTI, A.J., DUMONT, J.A. & MCCANN, P.P. 1986. Characterization of *Trypanosoma brucei brucei* S-adenosyl-L-methionine decarboxylase and its inhibition by Berenil, pentamidine and methylglyoxal bis(guanylhydrazone). *Biochemistry Journal*, 237:685–689.

HIRUMI, K., NELSON, R.T., HIRUMI, H., MOLOO, S.K. & NANTULYA, V. 1985. Characterization of *Trypanosoma vivax* metacyclic trypomastigotes propagated *in vitro*. *Proceedings, VII International Congress of Protozoology*, Abstract No. 46, Nairobi, Kenya.

KAMINSKY, R. & ZWEYGARTH, E. 1989. Feeder layer-free *in vitro* assay for screening antitrypanosomal compounds against *Trypanosoma brucei brucei* and *T. b. evansi*. *Antimicrobial Agents and Chemotherapy*, 33:881–885.

KAMINSKY, R., CHUMA, F., ZWEYGARTH, E., KITOSI, D.S. & MOLOO, S.K. 1991. The effect of isometamidium chloride on insect forms of drug-sensitive and drug-resistant stocks of *Trypanosoma vivax*: Studies *in vitro* and in tsetse. *Parasitology Research*, 77:13–17.

NEWTON, B.A. & LEPAGE, R.W.F. 1967. Preferential inhibition of extranuclear deoxyribonucleic acid synthesis by the trypanocide Berenil. *Biochemistry Journal*, 105.

RAYNAUD, J.P., SONES, K.R. & FRIEDHEIM, E.A.H. 1989. *A review of cymelarsan®—a new treatment proposed for animal trypanosomiasis due to* T. evansi *and other trypanosomes of the* T. brucei *group*. International Scientific Council for Trypanosomiasis Research and Control. Publication No. 115:334–338.

SCHÖNEFELD, A., RÖTTCHER, D. & MOLOO, S.K. 1987. The sensitivity to trypanocidal drugs of *Trypanosoma vivax* isolated in Kenya and Somalia. *Tropical Medicine and Parasitology*, 38:177–180.

VAN NIEUWENHOWE, S., SCHECHTER, P.J., DECLERCQ, J., BONE, G., BURKE, J. & SJOERDSMA, A. 1985. Treatment of gambiense sleeping sickness in the Sudan with oral DFMO (DL-alpha-difluoromethyl-ornithine), an inhibitor of ornithine decarboxylase; first field trial. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 79:692–698.

YABU, Y., TAKAYANAGI, T. & SATO, S. 1989. Long-term culture and cloning system for *Trypanosoma brucei* gambiense bloodstream forms in semi-defined medium *in vitro*. *Parasitology Research*, 76:93–97.

ZWEYGARTH, E. & KAMINSKY, R. 1990. Evaluation of an arsenical compound (RM 110, mel Cy, Cymelarsan®) against susceptible and drug-resistant *Trypanosoma brucei brucei* and *T. b. evansi*. *Tropical Medicine and Parasitology*, 41:208–212.

ZWEYGARTH, E., GRAY, M.A. & KAMINSKY, R. 1991a. Axenic *in vitro* cultivation of *Trypanosoma vivax* trypomastigote forms. *Tropical Medicine and Parasitology*, 42:45–48.

ZWEYGARTH, E., KAMINSKY, R. & GRAY, M.A. 1991b. *In vitro* assessment of isometamidium chloride susceptibility of *Trypanosoma vivax* bloodstream forms. *Parasitology Research*, 77:714–716.

ZWEYGARTH, E., MOLOO, S.K. & KAMINSKY, R. 1993. *Trypanosoma simiae*: *In vitro* studies on drug susceptibility. *Acta Tropica*, 54:301–308.