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Trypanocidal resistance in *Trypanosoma evansi* in vitro: effects of Verapamil, Cyproheptidine, Desipramine and Chlorpromazine alone and in combination with trypanocides

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Trypanocidal resistance in *Trypanosoma evansi* in vitro: effects of Verapamil, Cyproheptidine, Desipramine and Chlorpromazine alone and in combination with trypanocides

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Abstract

A study was conducted in vitro to assess the ability of calcium antagonists to reverse trypanocidal resistance in *Trypanosoma evansi*. Susceptibility patterns of sensitive and resistant parasites were evaluated against calcium antagonists of several chemical classes (verapamil, cyproheptidine, desipramine and chlorpromazine), alone and in combination with suramin, diminazene aceturate or melarsen oxide cyteamine. The putative resistance modulators were intrinsically antitrypanosomal, but were unable to reverse resistance to any of the trypanocides tested. It was thus concluded that resistance to these trypanocides in *T. evansi* may differ from drug resistance mechanisms occurring in cancer cells, malaria or in South American trypanosomiasis, where calcium antagonists have successfully reversed resistance.

Keywords: *Trypanosoma evansi*; Resistance; Verapamil; Cyproheptidine; Desopramine; Chlopromazine; Calcium antagonists

1. Introduction

With the spread of drug resistant parasites into all trypanosomiasis-endemic areas, development of new antitrypanosomal compounds and drugs to circumvent resistance is urgently needed. However, it appears unlikely that new compounds will be introduced in

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the near future because of lack of interest by the pharmaceutical industry in investing in research and development of antitrypanosomal drugs (Gutteridge, 1985). Hence the need to intensify efforts at maintaining the efficacy of existing trypanocides.

Multi-drug resistance (*mdr*) in cancer and malaria is mediated by over-expression of a membrane protein called *P*-glycoprotein responsible for enhanced drug efflux which prevents drug accumulation from reaching toxic levels (Fojo et al., 1985; Krogstad et al., 1987; Martin et al., 1987; Gottesman and Pastan, 1988, 1993). Several workers have shown that certain calcium antagonists including verapamil, desipramine, cyproheptidine and chlorpromazine were effective in reversing resistance in neoplastic cells (Gottesman and Pastan, 1988) and malaria parasites (Krogstad et al., 1987; Martin et al., 1987; Bitonti and McCann 1989; Peters et al., 1989; Tanabe et al., 1990). A similar mechanism of drug resistance is reportedly shared by some trypanosomatids. A correlation was established between the level of trypanocides accumulated by *T. brucei*-group trypanosomes (Damper and Patton, 1976; Frommel and Balber, 1987) and *T. congolense* (Sutherland and Holmes, 1993) and reduction in sensitivity to trypanocides. There have been reports of reversal of drug resistance in *T. cruzi* (Neal et al., 1989) and *Leishmania* spp. (Neal et al., 1989; Dey et al., 1994) by verapamil. Furthermore, Sutherland and Holmes (1993) showed that addition of verapamil and desipramine effectively blocked isometamidium chloride efflux from resistant *T. congolense*. However, no reversal of resistance was demonstrated in *T. brucei* by inclusion of verapamil with diminazene aceturate or isometamidium chloride (Kaminsky and Zwegarth, 1991) or with melarsen oxide (Yarlett et al., 1991).

In the light of these conflicting reports a more comprehensive investigation is required, to test a larger range of both trypanocides and putative modulators. We have investigated the effects of a number of calcium antagonists of several chemical classes (verapamil, desipramine, cyproheptidine and chlorpromazine) on *T. evansi* stocks grown in vitro which differ in their sensitivity to melarsen oxide cysteamine, diminazene aceturate and suramin.

2. Materials and methods

2.1. Trypanosomes and cultures

The *T. evansi* stocks used were: TREU 1981, a cloned derivative of TREU 1914, primary isolation code MINHASA/84/BAKIT/148; a derivative, 1981 MC, resistant to melarsen oxide cysteamine (Cymelarsan) and diminazene aceturate after induction of resistance to Cymelarsan in vitro; and TREU 2136, a cloned derivative of a camel isolate from Sudan and demonstrating a high level of resistance to suramin (Luckins et al., 1979). Trypanosomes were grown in vitro in Eagle's Minimum Essential Medium with Earle's salts (Gibco BRL, Uxbridge, UK) as described in Ross and Taylor (1994).

2.2. Drugs

Verapamil, desipramine, cyproheptidine and chlorpromazine were purchased from Sigma Chemical Company (Poole, UK). Melarsen oxide cysteamine (Cymelarsan) was

obtained from Rhone Merieux (Toulouse, Cedex, France) diminazene aceturate (Berenil) from Hoechst AG, Frankfurt, Germany, and suramin from Bayer AG, Luverkusen, Germany. Working solutions of these drugs except suramin (aliquots frozen) were prepared immediately before use, by appropriate dilution in distilled deionised water (DDW) and sterilised by filtration through a 0.22 μm pore-size filter. Stock solutions of each trypanocide (melarsen oxide cysteamine and diminazene aceturate at 1 mg ml⁻¹ or suramin at 100 mg ml⁻¹) were serially diluted in DDW and drug dilutions were added to trypanosome cultures at 1:100 (v/v). The verapamil stock solution at 20.4 $\times 10^6$ nM (10 mg ml⁻¹) was also diluted in DDW and drug dilutions were added at 1:100 (v/v). Other calcium antagonists were diluted from stock solutions of 10⁶ nM as in Basco and Le Bras (1990a,b). When combinations of drugs were used, each drug solution was added to the trypanosome suspension at 1:100 (v/v).

2.3. Growth inhibition assay

Assays were performed using subcultures of trypanosomes that were routinely maintained in vitro. Bijoux containing 1 ml samples of the trypanosome suspension at a concentration of 1 $\times 10^5$ and 2 $\times 10^5$ trypanosomes per ml for TREU 1981 and TREU 2136, respectively, were prepared and 10 μl of appropriate drug solution was added. Parasites grown in the absence of drug received an equivalent volume of drug solvent. Trypanosome suspensions containing drug or drug solvent were dispensed in 100 μl volumes to the wells of 96-well plates. Each drug concentration was assayed in duplicate, and assays repeated at least twice. After 40 h of incubation at 37°C in a humidified 5% CO₂ atmosphere, the extent of cell proliferation at each assay condition was measured by the Promega Cell Titer Assay Kit (Promega Corporation, Southampton, UK), essentially as described in Ross and Taylor (1994). Assays were performed to assess the effect of each calcium antagonist and each trypanocide alone, then later with trypanocide in the presence of selected concentrations of each modulator. The concentration of each drug or drug combination at which growth was inhibited by 50% compared with control trypanosomes incubated in the absence of drug (IC₅₀), was calculated. Assays were carried out three times and data presented as mean IC₅₀ \pm standard error.

3. Results

Initial experiments to assess the effect of the modulators alone showed that they were intrinsically toxic to the trypanosomes. The IC₅₀ of the modulators alone are shown in Table 1 and were similar in all three stocks for each modulator. The calculated IC₅₀s were of the order of micromolar (μM), and were least for chlorpromazine and highest for verapamil for all stocks. Subsequent concentrations of modulators were chosen within the non-toxic range for trypanosomes and combined with trypanocides.

Results of assays containing combinations of sub-lethal concentrations of modulators with trypanocides, to test their ability to reverse trypanocidal resistance in the parasites, are presented in Tables 2, 3, and 4 for suramin, diminazene aceturate and melarsen oxide cysteamine, respectively. The trypanosome stocks studied showed a profound variation

Table 1
Susceptibility of *T. evansi* to calcium antagonists

Drug	IC ₅₀ (nM)		
	TREU 1981 MC	TREU 1981	TREU 2136
Verapamil	8620.0 ± 1391.1	7728.3 ± 602.8	6767.1 ± 577.4
Cyproheptidine	5517.2 ± 957.2	6321.5 ± 894.4	5807.5 ± 1012.6
Desipramine	3436.5 ± 181.5	3363.1 ± 141.7	2963.5 ± 714.5
Chlorpromazine	1808.8 ± 245.7	1472.7 ± 158.3	1800.4 ± 227.0

Table 2
Influence of verapamil, cyproheptidine, desipramine and chlorpromazine on the response of *T. evansi* to suramin

Drug (nM)		IC ₅₀		
		TREU 1981 MC (ng ml ⁻¹)	TREU 1981 (ng ml ⁻¹)	TREU 2136 (ug ml ⁻¹)
Verapamil	0	31.3 ± 4.3	41.4 ± 5.7	20.6 ± 3.0
	203.6	28.4 ± 1.4	31.6 ± 0.0	19.0 ± 6.2*
	2036.0	34.8 ± 5.0	31.6 ± 0.0	16.7 ± 5.5
Cyproheptidine	0	65.5 ± 30.3	49.5 ± 10.9	24.5 ± 1.4
	312.5	58.4 ± 2.5	ND	21.8 ± 0.0
	2500.0	ND	55.9 ± 4.4	20.7 ± 3.4
Desipramine	0	39.5 ± 9.6	43.3 ± 9.9	22.5 ± 2.5
	312.5	39.8 ± 6.3	42.4 ± 10.9	21.8 ± 2.4
	1250.0	44.5 ± 6.9	52.4 ± 12.7	15.3 ± 4.7
Chlorpromazine	0	37.5 ± 0.5	34.6 ± 2.9	21.5 ± 1.8
	312.5	38.5 ± 1.5	24.1 ± 13.6	19.0 ± 2.1
	1250.0	39.8 ± 2.2	44.8 ± 34.3	12.1 ± 1.0

ND Not done.

Table 3
Influence of verapamil, cyproheptidine, desipramine and chlorpromazine on the response of *T. evansi* to diminazene aceturate

Drug (nM)		IC ₅₀ (ng ml ⁻¹)	
		TREU 1981 MC	TREU 1981
Verapamil	0	267.5 ± 28.0	3.7 ± 0.05
	2036.0	279.7 ± 50.9	3.5 ± 0.1
Cyproheptidine	0	350.0 ± 48.6	4.4 ± 0.9
	312.5	329.0 ± 27.0	3.5 ± 0.3
	2500.0	336.0 ± 19.0	3.5 ± 0.14
Desipramine	0	261.3 ± 37.6	3.4 ± 0.17
	312.5	237.6 ± 13.7	3.1 ± 0.21
	1250.0	245.1 ± 21.3	3.0 ± 0.17
Chlorpromazine	0	336.0 ± 38.0	5.0 ± 1.0
	312.5	334.0 ± 27.7	3.1 ± 2.1
	1250.0	329.0 ± 13.9	3.0 ± 2.1

Table 4

Influence of verapamil, cyproheptidine, desipramine and chlorpromazine on the response of *T. evansi* to melarsen oxide cysteamine

Drug (nM)		IC ₅₀ (ng ml ⁻¹)		
		TREU 1981 MC	TREU 1981	TREU 2136
Verapamil	0	136.5 ± 26.8	0.66 ± 0.34	ND
	203.6	116.0 ± 34.4	0.76 ± 0.44	ND
	2036.0	117.0 ± 28.3	1.0 ± 0.66	ND
Cyproheptidine	0	181.8 ± 85.0	2.3 ± 0.93	4.2 ± 0.24
	2500.0	162.0 ± 86.0	2.0 ± 0.86	3.3 ± 0.08
Desipramine	0	132.8 ± 79.3	0.52 ± 0.04	ND
	312.5	120.7 ± 74.3	0.50 ± 0.04	ND
	1250.0	116.5 ± 67.0	0.47 ± 0.01	ND
Chlorpromazine	0	55.1 ± 5.0	1.65 ± 1.5	5.9 ± 0.86
	312.5	54.3 ± 0.3	1.62 ± 1.6	5.7 ± 1.2
	1250.0	54.6 ± 1.6	1.54 ± 1.4	4.6 ± 1.8

ND Not done.

in their sensitivity to trypanocides. The TREU 2136 isolated from the field with suramin resistance showed an approximately 1000-fold higher IC₅₀ for suramin compared to both types of TREU 1981. A comparison of the IC₅₀ for suramin showed no differences in either resistant or sensitive stocks when assayed in the presence of modulators. Similar results were obtained when diminazene aceturate (Table 3) or melarsen oxide cysteamine (Table 4) were combined with modulators.

The process of induction of resistance to melarsen oxide cysteamine in TREU 1981 produced about 100-fold difference in the susceptibility between the two types of trypanosome, as well as an approximately similar level of cross-resistance to diminazene aceturate. The TREU 1981 MC with induced resistance to melarsen oxide cysteamine was sensitive to suramin. Similarly, TREU 2136 expressing a high level of resistance to suramin (15–26.6 µg ml⁻¹) was susceptible to melarsen oxide cysteamine (4–6.7 ng ml⁻¹) as well as to diminazene aceturate (36–89 ng ml⁻¹), implying that suramin does not share a similar pattern of resistance to melarsen oxide cysteamine and diminazene aceturate. Compared with suramin and diminazene aceturate, the IC₅₀ for melarsen oxide cysteamine varied considerably especially within each type of TREU 1981.

4. Discussion

The differential trypanocidal susceptibility between the *T. evansi* stocks used in this study were clearly expressed in the results. TREU 2136 was previously noted to express high levels of resistance to suramin (Luckins et al., 1979). The variation between experiments observed in the sensitivity of trypanosomes to melarsen oxide cysteamine may be associated with the instability of the drug in solution and the varying toxicity of the dissociation products formed to the parasites (Berger and Fairlamb, 1994).

All the modulators tested exhibited anti-trypanosomal activity and to the same extent

in each of the stocks. Chlorpromazine demonstrated anti-trypanosomal activity at the lowest concentration. Many tricyclic compounds have been noted previously to have anti-trypanosomal and anti-leishmanial activity of which chlorpromazine was found to be the most potent (Benson et al., 1992). The activity of these compounds is thought to arise from their selective inhibition of trypanothione reductase, a target common to all trypanosomatids (Hammond et al. 1985) as well as interference with micro-tubule assembly (Seebeck and Gehr, 1983) and membrane disruption especially of the proton based electrochemical gradient (Pearson et al., 1982; Zilberstein and Dwyer, 1984).

Important considerations for possible clinical application of a compound include its bioavailability and its toxicity at the concentration that must be achieved in the blood to produce a chemotherapeutic effect. In this study, the concentrations of verapamil, cyproheptidine and desipramine that inhibited growth of trypanosomes by 50% (IC_{50}) or more were considerably higher than their clinically achievable plasma level, which for verapamil was 400 nM (Watt et al., 1990), for cyproheptidine was 103–142 nM (Hintze et al., 1975) and for desipramine was 300 nM (Peters et al., 1989; Sjokvist, 1971). However, the IC_{50} obtained for chlorpromazine (850–2814.5 nM) did not differ remarkably from the therapeutic range of the drug (0.1–0.5 $\mu\text{g ml}^{-1}$ or 281–1407.3 nM) (Pearson et al., 1982). These observations suggest that chlorpromazine might constitute a promising candidate for rational drug design as a trypanocide. Indeed, it has been reported that structural modification of chlorpromazine by oxidation of the tricyclic ring produced a compound with greater activity against protozoa than chlorpromazine (Pearson et al., 1982).

The results of our study further show that combinations of melarsen oxide cysteamine, diminazene aceturate or suramin with any of the candidate resistance modulators did not lead to any differences in the susceptibility pattern between sensitive and resistant *T. evansi* stocks studied. Modulators at concentrations that were by themselves inactive on trypanosomes did not reverse resistance to any of the trypanocides. Similar results were recorded in *T. brucei* when combinations of verapamil and diminazene aceturate or isometamidium chloride were applied (Kaminsky and Zweygarth, 1991). Although the cellular and biochemical mechanisms of resistance to trypanocides has not been fully understood (Kinabo, 1993; Peregrine, 1994), one mechanism associated with trypanocidal resistance is the active extrusion of drugs leading to reduced accumulation of drug in resistant parasites compared with drug sensitive organisms (Neal et al., 1989; Yarlett et al., 1991; Sutherland and Holmes, 1993). The outcome of this present study implies that these drugs are not exported by *P*-glycoproteins in *T. brucei* and *T. evansi*. Calcium antagonists reversed *mdr* in cancer and malaria by competing for the drug-binding site of the *P*-glycoprotein, which enhanced drug accumulation (Foote et al., 1989; Wilson et al., 1989).

It may thus be concluded that the mechanisms of resistance to trypanocides in *T. evansi* and related *T. brucei* differ from *mdr* in cancer cells and malaria parasites, and indeed *T. cruzi* (Neal et al., 1989) and *Leishmania* (Neal et al., 1989; Dey et al., 1994) where verapamil effectively reversed drug resistance. The mechanism of drug resistance acquired by extracellular parasites such as *T. evansi* and *T. brucei* may be fundamentally different from those employed by intracellular life cycle stages of *T. cruzi* and *Leishmania* spp.

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