IDENTIFICATION OF DRUGS EFFECTIVE AGAINST *COWDRIA RUMINANTIUM* USING A MOUSE SCREENING SYSTEM

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ABSTRACT

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Heartwater is a tick-transmitted rickettsial infection of ruminants, caused by *Cowdria ruminantium*. The tetracyclines are the only compounds available for therapy of the disease. A screen, using mice infected with *C. ruminantium*, was developed and used to identify new compounds with potential for the control of heartwater. A series of di-4-methyl-thiosemicarbazones was shown to be highly active in the mouse model and their efficacy was confirmed in further trials in sheep infected with *C. ruminantium*. The mouse screen was shown to be simple to operate and reliably predictive of activity against heartwater. Ways in which the screen may be improved are suggested.

INTRODUCTION

The tetracyclines are the only group of compounds currently available for the therapy and prophylaxis of heartwater caused by the rickettsia *Cowdria ruminantium*. They are generally effective for both purposes, provided they are administered relatively early in the clinical disease syndrome (Weiss, Haig & Alexander, 1952; Haig, Alexander & Weiss, 1954; Uilenberg, 1981). In "long-acting" form, oxytetracycline has shown particular promise (Purnell, 1984). There are references in the literature to the partial efficacy of antifolate drugs, such as the sulphonamides (Neitz & Alexander, 1945) and to the dithiosemicarbazones (McHardy, Berger, Taylor, Farebrother & James, 1980; Van der Merwe, 1979), which, though highly effective, have not been developed. So while the tetracyclines are available and widely used they are not ideal and there is a clear need for new and fully effective drugs.

Why, apparently, are no new drugs forthcoming? There seem to be five main reasons:

1. General lack of awareness of the importance of heartwater as a disease.

2. The view of potential developers of new drugs, notably the pharmaceutical industry, that the financial return on sales of a new drug would not justify the cost of research and development.

3. The intractability of heartwater to therapy with drugs which are available for the treatment of other infections, with the notable exception of tetracyclines, or to other compounds which have been screened, of which only the thiosemicarbazones have shown any real promise.

4. Reliance on control of vector ticks with acaricides which is perceived as being an alternative approach to the problem.

5. The lack of suitable screening models in which to test candidate compounds cost-effectively, and our near total ignorance of the biochemistry of the causative organism, resulting in no leads as to which compounds should be screened, using limited resources.

The laboratory white mouse, infected with *Cowdria ruminantium*, (Du Plessis & Kümm, 1971; MacKenzie & Van Rooyen, 1981) now provides the basis of a drug screening system, and early results using this system have been reported (McHardy & MacKenzie, 1984). In this paper we will describe our results with the mouse model, and we will show that our results correlate well with results obtained in infected sheep, and in the field. We will also suggest ways in which the model may be improved so as to produce useful results in the future.

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Four strains of *Cowdria ruminantium* have been adapted to infect, and kill, laboratory mice:

1. Kümm strain (Du Plessis & Kümm, 1971)
2. Kwanyanga strain (MacKenzie & Van Rooyen, 1981).
3. Nonile strain (MacKenzie & McHardy, 1984).
4. Welgevonden strain (Du Plessis, 1985).

Our initial studies were made with the Kümm strain, kindly supplied by Dr J. L. du Plessis. Latterly we used the Kwanyanga strain, since it can be readily passaged between mice, sheep, cattle and ticks. Little work has been done with the Nonile or Welgevonden strains.

MATERIALS AND METHODS

The Mouse Model

Groups of mice were infected by the intravenous (i.v.) injection of $0,1$ mℓ of the supernatant fluid of a homogenate in saline of liver from an acutely-ill mouse, infected with a mouse-adapted strain of *Cowdria* (Du Plessis & Kümm, 1971; McHardy & MacKenzie, 1984). The time to death in groups of 10 of each of 5 inbred (Balb/C, CBA/CA, C3H/HE, C57/BL6 and DBA2) and 1 randomly bred (CD-1) strains of mice, was compared (Tables 1 and 2). The mice were all male and mass-measured 15–20 g. We selected the Kwanyanga strain in Balb/C mice as the most consistent model, since all 20 mice died on day 9 (Table 2). We speculated that the consistency in time to death of all 6 strains of mice infected with the Kümm strain (Table 1) could reflect its far longer history of being passaged between mice.

TABLE 1 Lethality of the Kümm strain of *Cowdria ruminantium* in 6 strains of laboratory mice

Mouse strain	Mortality /10	Days to death
Balb/C	10	$9,7 \pm 0,50$
CBA/CA	10	$9,6 \pm 0,50$
C3H/HE	10	$10,0 \pm 0,00$
C57/BL6	10	$10,1 \pm 0,32$
DBA/2	10	$9,6 \pm 0,50$
CD-1	10	$9,2 \pm 0,42$

TABLE 2 Lethality of the Kwanyanga strain of *Cowdria ruminantium* in 6 strains of laboratory mice

Mouse strain	Trial 1		Trial 2	
	Mortality /10	Days to death	Mortality /10	Days to death
Balb/C	10	$9,0 \pm 0,00$	10	$9,0 \pm 0,00$
CBA/CA	10	$12,1 \pm 1,10$	10	$9,7 \pm 0,48$
C3H/HE	10	$11,8 \pm 1,23$	10	$10,4 \pm 0,52$
C57/BL6	10	$11,5 \pm 0,71$	10	$10,7 \pm 0,67$
DBA/2	10	$11,2 \pm 0,92$	10	$12,0 \pm 1,25$
CD-1	8	$12,6 \pm 1,60$	7	$12,1 \pm 2,48$

For use as a drug screen, the mice were again infected by the intravenous injection of 0,1 ml of infected mouse-liver supernatant. Seven days later, when the mice were still apparently healthy, groups of 10 were injected subcutaneously (s.c.) with an aqueous solution or micronized aqueous suspension of the trial compound, initially at 20 mg/kg body mass. The mice were observed twice daily and the time to death was recorded. About 500 compounds, representing about 100 chemical types, were tested in this way. Among them were compounds with known activity against a wide range of protozoa, bacteria, viruses and helminths as well as a range of novel structures from the Burroughs Wellcome and Company's collection of compounds. This compound bank contained a large number of carbazones and thiosemicarbazones. About 50 were selected for testing, and some new analogues were also synthesized and tested.

Using mice infected with the Kümm strain, a range of doses of oxytetracycline hydrochloride, the dithiosemicarbazone, gloxazone, and the carbanilide, imidocarb dipropionate was tested.

The 3 compounds, 216C, 235C and 272C, were then tested in sheep infected with the Ball 3 strain of *Cowdria ruminantium*. This is the strain, maintained by blood passage between sheep, commonly used as a "vaccine" strain in Southern Africa. It is capable of killing infected sheep in 12–16 days if the infection is not treated.

RESULTS

Screening in mice

A significant delay in time to death was observed only in mice treated with either tetracyclines or thiosemicarbazones. No other compounds possessed even marginal levels of activity. Three compounds, all of them di-4-methyl-thiosemicarbazones, were found to be particularly active when tested in the mouse-*Cowdria* model, using the Kwanyanga strain. Their activities were similar to those of gloxazone and oxytetracycline. Gloxazone and oxytetracycline were about equally active, and are known also to be active against heartwater. Imidocarb was totally inactive. Imidocarb is known not to be effective against heartwater but is active against *Anaplasma* and *Ehrlichia*, which are, taxonomically, related to *Cowdria*. The results are shown in Table 3.

Tests in Sheep

Fifteen Dorper yearlings were infected by the intravenous injecton of 5 ml of blood from a similar lamb clinically sick with the Ball 3 strain of *Cowdria ruminantium*. On the day on which the temperature of individual lambs rose above 40,5 °C, groups of 3 were treated by the i.v. injection of each of the three di-4-methyl-thiosemicarba-

TABLE 3 Efficacy of oxytetracycline hydrochloride, gloxazone and imidocarb diproprionate, injected subcutaneously, in mice infected with the Kümm strain of *Cowdria ruminantium*

Compound	Dosage (mg/kg)	Survival to day 18 Groups of 10 mice
Oxytetracycline	100	10
	50	9
	20	7
	10	4
	5	0
	2,5	0
Gloxazone	100	10
	50	10
	20	5
	10	4
	5	0
	2,5	0
Imidocarb	100–2,5	0
Untreated controls	—	0

TABLE 4 Efficacy of gloxazone and 3 di-4-methyl-thiosemicarbazones, injected intravenously, in Dorper lambs infected with the Ball 3 strain of *Cowdria ruminantium*

Treatment (Groups of 3)	Days to 40,5 °C (Day T)	T° at day T	Max. T° increase post day T (°C)	Days from T to Death	Days from T to Recovery
Untreated	9,3	40,8	1,2	4,0	—
Gloxazone 5,0 mg/kg	9,3	41,1	0,7	—	7,0
216C, 2,5 mg/kg	10,0	41,0	0,5	—	5,0
235C, 2,5 mg/kg	9,7	40,9	0,2	—	6,0
272C, 2,5 mg/kg	9,7	41,1	0,0	—	6,7

TABLE 5 Efficacy of gloxazone, oxytetracycline hydrochloride and 3 di-4-methyl-thiosemicarbazones injected intravenously in Merino lambs infected with the Ball 3 strain of *Cowdria ruminantium*

Treatment (Groups of 3)	mg/kg	T° at day 10 (day T)	Max. T° increase post day T (°C)	Days from T to Death	Days from T to Recovery
Untreated		40,8	1,1	4,7	—
Gloxazone	2,0	40,8	0,6	—	3,7
Oxytetracycline	8,0	40,7	0,5	—	4,0
216C	3,0	41,3	0,4	—	5,0
	2,0	40,6	0,9	—	4,7
	1,0	40,7	0,9	6(1)	4,5(2)
235C	3,0	41,6	0,0	—	3,3
	2,0	41,2	0,1	—	3,7
	1,0	41,0	0,4	—	5,0
272C	3,0	41,2	0,6	—	2,3
	2,0	40,4	0,7	—	4,7
	1,0	40,7	0,7	—	4,7

zones at 2,5 mg/kg or gloxazone at 5 mg/kg. A further group of 3 infected lambs was kept untreated, as controls. The results are shown in Table 4.

All 12 treated lambs were cured while all 3 controls died of heartwater. A lamb was regarded as cured when its temperature had fallen below 40,5 °C and no other symptoms of heartwater were evident. Although the most rapid cure was observed following treatment with 216C (mean 5,0 days after treatment), pyrexia following treatment was better controlled by 235C and 272C. Gloxazone, although injected at twice the dose rate, cured more slowly and permitted a greater pyrexia than any of the 3 new compounds.

Encouraged by this result, we tested each of the di-4-methyl-thiosemicarbazones at 3, 2 or 1 mg/kg in comparison with gloxazone at 2 mg/kg and oxytetracycline at 8 mg/kg, (a common field-use dosage) and an untreated, infected control group. The design of the experiment was as before except that more susceptible Merino lambs were used. All treatments were given on day 10, when all but 1 lamb had a temperature of 40,5 °C or higher. The results are shown in Table 5.

Compound 235C was selected as being the most active compound. Only 1 treated lamb died. It had received 216C at 1 mg/kg. All 3 untreated controls died.

In the final trial, Dorper lambs were infected with the virulent Kwanyanga strain, and 235C was used at 5 mg/kg, by either the i.v. or oral route, in groups of 9 lambs. Six lambs were infected but untreated, as controls. The results are shown in Table 6.

Three control lambs died within a mean of 4,3 days after temperature rose above 40,5 °C. The other 3 recovered in 7 days. All the treated lambs were cured, in about 2,5 days, indicating that 235C is similarly effective by the i.v. and oral routes. No signs of drug toxicity were observed in any of the sheep trials.

TABLE 6 Efficacy of the di-4-methyl-thiosemicarbazone 235C, administered by either the intravenous or oral route, in Dorper lambs infected with Kwanyanga strain of *Cowdria ruminantium*

Treatment	Day T	T° at day T	Max. T° increase post day T (°C)	Days from T to	
				Death	Recovery
Untreated (6 lambs)	8,7 ±0,56	41,1 ±0,18	0,6 ±0,16	4,3(3) ±0,88	7,0(3) ±2,0
235C 4,0 mg/kg i.v. (9 lambs)	8,4 ±0,24	40,9 ±0,13	0,7 ±,12	—	2,5 ±0,38
235C 4,0 mg/kg p.o. (9 lambs)	8,4 ±0,24	40,7 ±0,10	0,5 ±0,11	—	2,3 ±0,24

CONCLUSIONS

1. The mouse/*Cowdria ruminantium* model is a valid system in which to screen potential new compounds for control of heartwater.
2. Either the Kümm or Kwanyanga strain of mouse-adapted *Cowdria ruminantium* can be used, and the preferred strain of mouse is Balb/C.
3. The screen successfully identified the activity of oxytetracycline and gloxazone, which were previously known to be effective in the treatment of heartwater in ruminants. It also successfully registered a lack of activity among a large numer of other compounds known to have no activity or not expected to show such activity against heartwater.
4. The screen identified 3 new compounds, not previously tested against heartwater but related to gloxazone, as having high activity. Their activity was confirmed by subsequent testing in an infected sheep model.

DISCUSSION

The mouse screen has the merits of being simple to operate, relatively inexpensive and, apparently, accurate. Used as described here, when compounds were initially tested at 20 mg/kg, the screen can provide a valid result with a sample of only about 50 mg of trial compound.

Deficiencies in the model are relatively minor but include the need to infect the mice by i.v. injection of a supernatant of infected liver homogenate. Intravenous injection of mice does require a degree of skill and any large particles in the inoculum can cause rapid death of the mice. Infected mouse blood can be used but gives less uniform results over successive passages. The liver of one mouse is adequate to infect about 100 mice, but 0,1 mℓ of blood is required to obtain uniform lethality.

Death, and time to death, of the mice are the only reliable parameters by which to judge the outcome of the trial. This is clearly not a very sensitive end-point, although it proved adequate. Clinical symptoms of the infection-lethargy, piloerection and hunched-back were generally not seen until, at most, 24 h before death. As described in another paper presented at this symposium by P.K.I. MacKenzie, there is a marked depression of rectal temperature, and no pyrexia in the mice, beginning about 3 days before death. This could be used as an additional parameter. It is well known that it is extremely difficult to demonstrate the presence of *Cowdria* organisms in mice—we were able to find them, with difficulty, only in smears of peritoneal fluid, in cells which appeared to be macrophages, but Du Plessis & Kümm (1971) also found them in various tissues of out-bred Swiss mice infected with the Kümm strain.

This inability to monitor accurately the numbers of organisms in the mice is, clearly, a significant defect in the model. Detailed post-mortem examination, including histopathology and thin-section electron microscopy, in our 6 strains of mice which had died following infection with the Kwanyanga strain, failed to demonstrate any consistent lesion or localization of organisms.

Despite these defects, the model is workable and apparently gives valid results. No doubt the screen could be modified, so as to be more sensitive and perhaps less cumbersome. The liver homogenate retains its infectivity, though at a somewhat reduced level, when stored at −70 °C. Thus a bank of stabilate could be laid down to provide a uniform infective dose in successive tests. Group sizes of mice could be reduced, perhaps to five, particularly if confirmatory tests were to be run. An initial dose higher than 20 mg/kg, or multiple doses, could increase the chance of detecting marginal activity, thus increasing the chance of detecting "lead" active compounds. Similarly, testing each compound at more than one dose level could aid ranking of activity of compounds and give some indication of the potential toxicity of compounds. Sensitivity of the test could be improved by ensuring maximum bioavailability of compounds following administration. This can be done by dissolving compounds, wherever possible, in acceptable solvents, e.g., ethanol or dimethyl formamide, and diluting with saline before administration. If suspensions have to be used, they should be micronized, stable and homogeneous to ensure that all mice in a group receive a similar dose. Various suspending agents, such as Celacol, can be used.

Administration of compounds by a variety of routes, e.g., s.c., i.v., or oral, will increase the change of maximizing bioavailability and avoiding metabolism of candidate compounds. Despite this some inactive compounds may require to be metabolized to produce active derivatives.

Overall, the screen as we ran it was adequate but improvements could easily be made to increase its flexibility and sensitivity. The ultimate improvement—of assaying the number of organisms in the mice—appears to be unattainable at present.

There is an obvious need for a new product to control heartwater. For the present, the mouse screen seems to be the best and most economical tool to aid in its discovery, although an *in vitro* system, if attainable, would ultimately be preferable. We have no leads as to which compound types to screen first. The initial, and most obvious step would be to screen as many compounds as possible with known biological activity—whether against infective organisms or enzyme systems, or any with pharmacological activity. Known toxicity or difficulty of synthesis should not be a bar in the first instance. It may prove possible to synthesise acceptable, active analogues. The search may be long and tedious, or an active compound may be just around the corner. Comfort can be taken from the uncannily frequent observation that within a chemical series, the first compound tested is among the most active.

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