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Therapeutic action of MK-436 (2,5-nitroimidazole) on *Trypanosoma cruzi* infections in mice: a parasitological, serological, histopathological, and ultrastructural study*

S. G. ANDRADE,[1] R. C. SILVA,[2] C. M. G. SANTIAGO,[2] & L. A. R. FREITAS[3]

The anti-protozoal drug MK-436 (3-(1-methyl-5-nitroimidazol-2-yl)-3a,4,5,6,7,7a-hexahydro-1,2-benzisoxazole) was found to be effective against Trypanosoma cruzi *infections in mice (2 daily doses of 250 mg per kg body weight). Parasitaemia disappeared within 24 hours of treatment which was commenced during the early or late stages of acute infection. Intracellular* T. cruzi *parasites were also affected by the drug, ultrastructural findings showing severe cytoplasmic vacuolization and membrane alterations. Positive serological responses persisted in the majority of treated and parasitologically cured mice in the study. Cure rates varied from 72% to 100% and were similar regardless of the* T. cruzi *strain used (Y strain, type I; 12 SF strain, type II; or Colombian strain, type III). However, the proportion of positive serological tests and the frequency of inflammatory lesions were greatest for mice that were infected with the Colombian strain of the parasite.*

Several drugs have been used to treat *Trypanosoma cruzi* infections, but are either non-curative or toxic, as recently reviewed by Brener (*1*). At present, only nitrofurans (e.g., nifurtimox) and an imidazole derivative (benznidazole) are being used therapeutically to treat such infections. However, the cure rate with these drugs is low and drug-related adverse effects are frequent (*1*). Both of these drugs produce variable cure rates in mice, depending on the strain of *T. cruzi*, some of which are highly drug-resistant (*2*). Other drugs that have been investigated include allopurinol and ketoconazole. Allopurinol has been tested in mice and shown to suppress parasitaemia (*3*), but exhibits no curative effects in the treatment of humans with acute Chagas' disease (*4*). More recently, Meirovich et al. administered allopurinol to chronically infected patients and reported a high proportion of negative xenodiagnoses (*5*). Ketoconazole has also been investigated in acutely infected mice (*6*), and, although protecting the animals against infection with the Y strain of *T. cruzi*, did not cure them.

Malanga et al. (*7*), who investigated several 2-substituted 5-nitroimidazole derivatives, reported considerable differences in their efficacies against *T. cruzi* infections that depended on slight modifications to their structures. Of these compounds, 3-(1-methyl-5-nitroimidazol-2-yl)-3a,4,5,6,7,7a-hexahydro-1,2-benzisoxazole (MK-436), was the most effective at controlling parasitaemia. Murray et al. (*8*) stressed the effectiveness of nitro-heterocyclic compounds, including nifurtimox and benznidazole, against protozoa, and demonstrated a significant curative effect in both early and late infections caused by the Y strain of *T. cruzi*.

Here, we report the curative effect of MK-436 for mice infected with strains of *T. cruzi* that exhibit variable degrees of resistance to nifurtimox and benznidazole (*9–11*). Also the *in vivo* activity of the drug on intracellular parasites was investigated by electron microscopy. Finally, the study examined the therapeutic effect of administering MK-436 during the early or late stages of acute infection and the influence of the duration of treatment on the cure rate.

* From the Federal University of Bahia, Centro de Pesquisas Gonçalo Moniz, Rua Valdemar Falcão, 121-Brotas Salvador, Bahia, Brazil.

[1] Associate Professor. Requests for reprints should be sent to this author.

[2] Biologist.

[3] Assistant Professor.

MATERIALS AND METHODS

The study was carried out on 450 outbred Swiss mice of both sexes (weight range: 18–20 g), which were divided into three groups of 150 animals each. Each group was infected with a different strain of *T. cruzi*, as follows: group 1, with the Y strain; group 2, with the 12 SF strain; and group 3, with the Colombian strain. These strains are representative of types I, II, and III, respectively (*12*, *13*) and differ in their biological behaviours (*13*) and response to chemotherapy (*2*). Mice were injected intraperitoneally with an inoculum consisting of 50×10^4 blood trypomastigotes obtained from infected mice.

Each of the three groups was divided into the following four subgroups: 20 mice that were used to investigate the intracellular action of MK-436 and were killed from 4 to 72 hours after being given the first dose of the drug (10 mice were treated and 10 were used as untreated controls); 50 mice that were treated only during the early acute phase of infection; 50 mice that were treated only during the late acute phase; and 30 infected mice that served as untreated controls.

Treatment

MK-436[a] was wetted, as described by Murray et al. (*8*), by addition of the minimum amount of a mixture of Tween 80 and ethanol (1:1) and then suspended in distilled water (10 mg MK-436 per ml water). Schedule of treatment: two daily doses of 250 mg MK-436 per kg body weight, administered by gavage, for 10 days, for half the mice and 60 days for the other half.

Evaluation of parasitological tests

Parasitological cure tests (direct examination of blood, inoculation of blood into newborn mice, and xenodiagnosis with fourth- to fifth-stage nymphs of *Rhodnius prolixus* 30–90 days after treatment) were carried out on the treated mice. Surviving mice were tested in the same way and also with a haemoculture test (Warren medium) 6–10 months after treatment.

Serological evaluation

Indirect immunofluorescence (IIF) tests were performed as described by Camargo (*14*), using culture forms of *T. cruzi* with serum dilutions of 1:10, 1:20, 1:40, and 1:80; anti-mouse IgG fluorescein conjugate was used at a dilution of 1:80. Complement-mediated lysis tests (CML) were carried out, as described by Krettli & Brener (*15*), on surviving mice that were negative in the parasitological tests performed 6–10 months after drug treatment. CML tests were considered positive if the lysis level reached 20% or more.

Histopathological study

Animals in the subgroups of 20 mice used to study the intracellular effect of MK-436 were killed 4, 8, 24, 48, and 72 hours, respectively, after administration of the initial dose; and mice in the other subgroups were killed 4, 7, 15, 20, and 30 days, respectively, after the beginning of treatment. Survivors were killed 6 to 10 months after the treatment had finished; the untreated controls in each group were also killed. All the mice were autopsied: sections of tissues were fixed in 10% formalin and embedded in paraffin, while sections of 5 μm thickness that had been stained with haematoxylin and eosin were used for histopathological studies.

Electron microscopy

Two sets of three mice that were infected with the 12 SF strain of *T. cruzi* were killed 6 and 24 hours, respectively, after the initial dose of MK-436, together with six untreated and infected controls. The mice were dissected and sections of the hearts immediately fixed in a mixture of 2% glutaraldehyde in sodium cacodylate buffer for 1 hour and then post-fixed with osmium tetroxide in 0.15 mol/l sodium cacodylate buffer. Preparations were dehydrated by treatment with ethanol followed by propylene oxide and embedded in Epon. Ultrathin sections, prepared using an ultramicrotome,[b] were treated with uranyl acetate–lead citrate contrast solution, and examined in the electron microscope[c] at 50 kV.

RESULTS

The parasitaemia curves for treated and untreated mice infected with the Y, 12 SF, and Colombian strains of *T. cruzi* are shown in Fig. 1, 2, and 3, respectively. Parasitaemia disappeared within 24 hours of treatment, both in the early and late stages of acute infections. The mortality rates in Table 1 vary, depending on the particular strain of the parasite. Mice infected with the Colombian strain that were treated from the 7th day to the 10th day of infection

[a] Merck, Sharp & Dohme Research Laboratories, P.O. Box 2.000, Rahway, NJ 07065, USA.

[b] Reichert ultramicrotome. C. Reichert AG. Hernalseq, Haupstrasse 219, A-1170 Vienna, Austria.

[c] Zeiss EM-109 model. Carl Zeiss, D-7082 Oberkochen, Federal Republic of Germany.

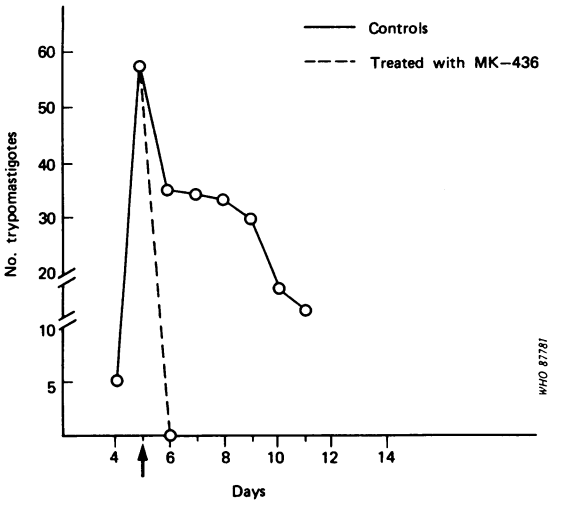

Fig. 1. Parasitaemia in mice infected with the Y strain of *Trypanosoma cruzi* and treated with MK-436; treatment begun on the 5th day of infection (50 fields, ×400).

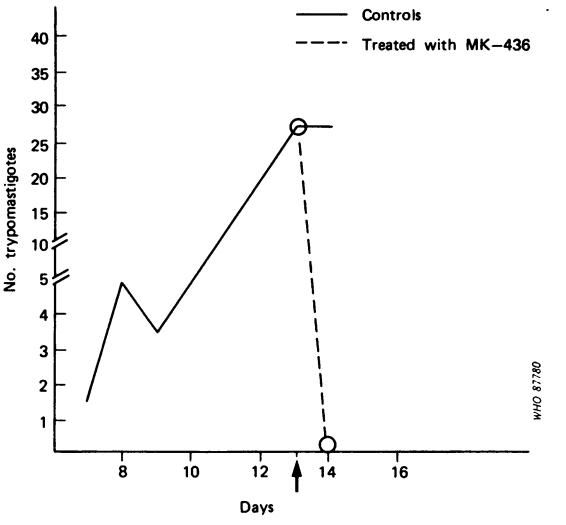

Fig. 2. Parasitaemia in mice infected with the 12 SF strain of *Trypanosoma cruzi* and treated with MK-436; treatment begun on the 13th day of infection (50 fields, ×400),

Table 1. Results of treating mice acutely infected with three different strains of *Trypanosoma cruzi* with MK-436

Experimental group	Strains of *Trypanosoma cruzi*	No. of mice[a]	Day of infection when treatment began	Duration of treatment (days)	Mortality rate[b] (until 90 days) (%)	Parasitological cure rate[c] (%)
I	Y	50	5th	10	55	88
				60	79	100
		50	8th	10	10	72
				60	16	100
II	12 SF	50	5th	10	34	100
				60	48	100
		50	14th	10	29	100
				60	48	100
III	Colombian	50	7th	10	0	80
				60	27	100
		50	20th	10	67	100
				60	48	100

[a] Each experimental group included also 30 untreated control mice and 20 mice used to study the intracellular action of the drug.
[b] Mortality of the untreated controls was 100%.
[c] Evaluated for the surviving mice 30 to 90 days and 6 to 10 months after treatment.

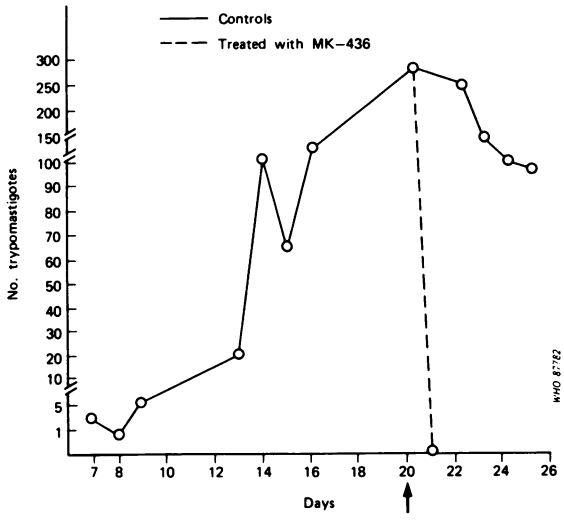

Fig. 3. Parasitaemia in mice infected with the Colombian strain of *Trypanosoma cruzi* and treated with MK-436; treatment begun on the 20th day of infection (50 fields, ×400).

exhibited the highest survival rate. The results of the parasitological cure tests (Table 1) ranged from 72% to 100%.

Table 2. Proportion of positive serological tests among mice acutely infected with *Trypanosoma cruzi* and treated with MK-436[a]

Experimental group	*Trypanosoma cruzi* strains	Serological test: Indirect fluorescence (% positive)	Serological test: Complement-mediated lysis (% positive)
I	Y	73	18
II	12 SF	64.5	28.5
III	Colombian	79.4	40.0

[a] All mice were parasitologically negative and were evaluated 6–10 months after the end of treatment.

Serological examinations

Table 2 shows the proportion of positive CML and IIF tests for mice that were parasitologically negative. Tables 3, 4, and 5 show, for mice in groups I, II, and III, respectively, the titres in the IIF test and the proportion of lysis as well as the correlation of these results with the histopathological lesions in parasitologically negative mice that were killed 6–10 months after treatment.

Table 3. Parasitological and serological evaluation of mice (group I) infected with the Y strain of *Trypanosoma cruzi* and treated with MK-436[a]

Identification	Duration of the infection (months)	Serological test: Indirect immunofluorescence	Serological test: Complement-mediated lysis (%)	Parasitological tests	Histopathology of the myocardium
Y-MK-54	10	1:1	54.3	−ve	Focal inflammatory infiltration (+)
Y-MK-56	10	−ve	10	−ve	Focal inflammatory infiltration (+)
Y-MK-57	10	−ve	12	−ve	Focal inflammatory infiltration (+)
Y-MK-59	10	1:10	19	−ve	Focal inflammatory infiltration (+)
Y-MK-60	7	1:40	0	−ve	NA[b]
Y-MK-61	7	1:20	0	−ve	NA
Y-MK-62	7	1:40	0	−ve	NA
Y-MK-63	7	1:40	0	−ve	NA
Y-MK-64	7	1:40	33	−ve	NA
Y-MK-65	7	1:40	18	−ve	NA
Y-MK-66	7	1:20	3	−ve	NA

[a] Animals that survived for 6 to 10 months after the end of treatment and were parasitologically negative.
[b] NA = no alteration.

Table 4. Parasitological and serological evaluation of mice (group II) infected with the 12 SF strain of *Trypanosoma cruzi* and treated with MK-436[a]

Identification[b]	Serological test		Parasitological tests	Histopathology of the myocardium
	Indirect immunofluorescence	Complement-mediated lysis (%)		
12 SF-MK-31	1:10	16.2	−ve	NA[c]
12 SF-MK-32	1:10	69.0	−ve	NA
12 SF-MK-33	1:10	26.0	−ve	NA
12 SF-MK-34	1:10	0	−ve	NA
12 SF-MK-35	1:20	0	−ve	NA
12 SF-MK-36	1:40	0	−ve	NA
12 SF-MK-48	−ve	51.0	−ve	Focal inflammatory infiltration (+)

[a] Animals that survived for 6 to 10 months after the end of treatment and were parasitologically negative.

[b] The duration of the infection was 6 months for all mice.

[c] NA = no alteration.

Ultrastructural studies

Six hours after administering the first dose of MK-436, the cytoplasm of intracellular amastigotes exhibited microvacuoles, small myelin-like bodies, and, occasionally, lipidic droplets. Parasites were irregularly shaped and early myocell lysis was observed (Fig. 4). Twenty-four hours after the first dose, most of the intracellular parasites had undergone marked structural alteration with considerable intensification of the previously mentioned changes (Fig. 5). The endoplasmic reticulum vesicles of non-parasitized cardiac myocells were dilated (Fig. 6).

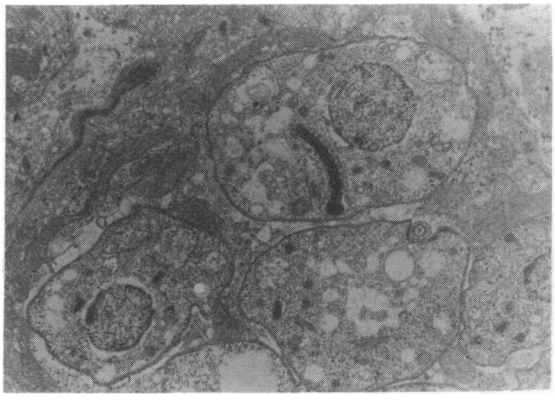

Fig. 4. Ultrastructure of intracellular amastigote forms of *Trypanosoma cruzi* 6 hours after beginning treatment with MK-436; cytoplasmic vacuolization and membrane irregularities can be observed (×7000).

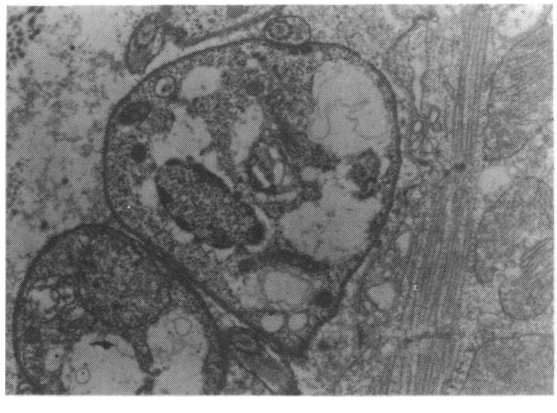

Fig. 5. Amastigote forms of *Trypanosoma cruzi* showing intense cytoplasmic vacuolization and lipid droplets 24 hours after the first dose of MK-436 (×12 000).

Table 5. Parasitological and serological evaluation of mice (group III) infected with the Colombian strain of *Trypanosoma cruzi* and treated with MK-436[a]

Identification	Duration of the infection (months)	Serological test: Indirect immunofluorescence	Serological test: Complement-mediated lysis (%)	Parasitological tests	Histopathology of the myocardium
Colom-MK-49[b]	9	1:10	43	−ve	Diffuse inflammatory infiltration (++)
Colom-MK-50	9	1:1	22	−ve	Focal inflammatory infiltration (+)
Colom-MK-53	9	1:1	17	−ve	Focal inflammatory infiltration (+)
Colom-MK-60	10	1:40	0	−ve	NA[c]
Colom-MK-61	10	1:20	0	−ve	NA
Colom-MK-62	10	1:40	2	−ve	NA
Colom-MK-56	10	1:10	7	−ve	NA
Colom-MK-57	10	1:10	33	−ve	Diffuse inflammatory infiltration (++)
Colom-MK-58	10	1:10	0	−ve	NA

[a] Animals that survived for 6 to 10 months after the end of treatment and were parasitologically negative.
[b] Two mice of this group (not included) and with positive xenodiagnosis showed diffuse inflammatory infiltration (++).
[c] NA = no alteration.

Histopathological studies

Between 4 and 8 hours after the beginning of treatment, the cardiac myocells of mice infected with each strain of *T. cruzi* showed the same degree of parasitism as those of the untreated controls. For mice infected with the Y and 12 SF strains, parasites disappeared 24–48 hours after the treatment had begun. Intracellular parasites of the Colombian strain persisted for 24–48 hours after the beginning of treatment but disappeared within 72 hours.

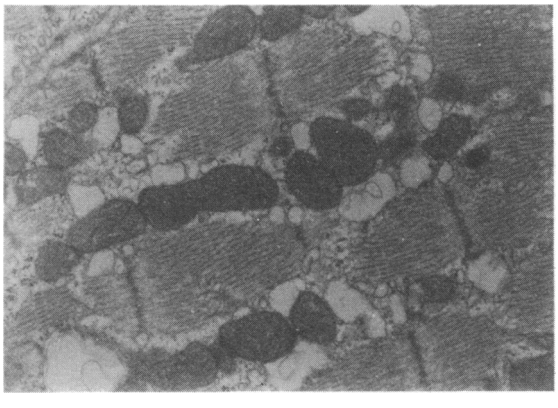

Fig. 6. Non-parasitized cardiac myocell from mice infected with *Trypanosoma cruzi* and treated with MK-436. Intense dilatation of the endoplasmic reticulum vesicles can be seen (×12 000).

On the 4th, 7th, 15th, and 30th days after treatment mice that had received the drug at an early phase of the acute infection had no parasites or inflammatory infiltrations. Ninety days after treatment, 1 out of 7 mice infected with the Y strain showed focal inflammatory infiltration of the myocardium. In contrast, mice that were infected with the 12 SF strain and survived for 10 to 12 months after treatment did not display tissue parasitism, and only 1 out of 10 exhibited moderate focal infiltration of the myocardium. Of the 9 mice infected with the Colombian strain, 4 exhibited focal and mild mononuclear infiltration of the myocardium, 2 demonstrated diffuse mononuclear infiltration, while 2 cases showed necrotizing anteriolitis in the skeletal muscles.

Four days after treatment had begun, mice administered MK-436 in the late phase of the acute infection had no parasites, but showed more intensified inflammatory infiltration than untreated controls. When examined at 6 days (Y strain) and 15 days (12 SF and Colombian strains), respectively, after treatment had begun, infected mice exhibited reduced infiltration and parasitism. In follow-up studies from 6 to 10 months after treatment, mild focal mononuclear infiltrates were observed in 7 out of 14 mice infected with the Y strain; in 1 out of 13 infected with the 12 SF strain; and 1 out of 5 infected with the Colombian strain.

In Tables 3, 4, and 5, respectively, the histopathological lesions are correlated with the results of the IIF and CML tests that were performed on parasitologically negative mice for each of the three strains of

T. cruzi. The titres in the IIF test varied from zero to 1:40, and no correlation was observed between the titres for specific antibodies and the histopathological lesions.

Complement-mediated lysis tests were positive ($\geqslant 20\%$) in 2 out of 11 mice infected with the Y strain; in 3 out of 7 infected with the 12 SF strain; and in 3 out of 9 infected with the Colombian strain. For those infected with the Y or 12 SF strain there was no correlation between a positive CML test and the presence of inflammatory infiltrations; however, for mice infected with the Colombian strain, a positive CML test correlated with the presence of moderate inflammatory infiltration, comparable to the histopathological lesions seen in two parasitologically positive animals (Table 5).

Table 6. Parasite clearance rates for mice infected with various strains of *Trypanosoma cruzi* with benznidazole or nifurtimox[a]

Trypanosoma cruzi strain	Biological type	Parasite clearance rate (%)	
		Benznidazole	Nifurtimox
Y	I	50	35
12 SF	II	100	100
Colombian	III	16.7	0

[a] See Andrade et al. (*2*).

DISCUSSION

MK-436 had a significant effect on parasitaemia in mice infected with different strains of *T. cruzi* parasites; the latter were cleared from peripheral blood 24 hours after treatment had begun. Reduction of intracellular parasitism was also observed, occurring earlier for infections with the Y and 12 SF strains than for those with the Colombian strain. This reduction was observed electron microscopically as early as 6 hours after the drug had been given.

Destruction of intracellular parasites is consistent with the results of ultrastructural studies, and similar to that already described for the nitrofuranic compounds (*16*, *17*). Subsequently, lesions of the intracellular organites (*19*) and intensification of the inflammatory process occurred (*16*).

Irrespective of parasite strain, histopathological lesions and tissue parasitism were not detected in the mice examined 4 days after the initial dose of MK-436. These results confirm observations made by Murray et al. (*8*) on the effect of treatment in the early phase of *T. cruzi* infections.

Mice treated with MK-436 had a lower mortality rate than infected, untreated controls, all of which died (from 10 days after infection for the Y strain to 50 days for the Colombian strain). No differences were noted in this respect for mice treated during the early or late phases of acute infection, with the exception of those infected with the Colombian strain. The latter mice, which were treated from the 7th day of infection, survived longer than those treated from the 20th day. Early mortality in treated mice occurred up to 90 days after treatment and was attributed to intensification of myocarditis in those treated with the drug during the late phase of acute infection. Other intercurrent factors, such as bronchopneumonia due to aspiration during gavage, also occurred.

Irrespective of the strain of *T. cruzi* with which mice were infected, parasitological clearance rates were independent of the duration of treatment with MK-436. The Colombian strain (type III) showed significant susceptibility to MK-436, although type III strains of the parasite are resistant to treatment with benznidazole and nifurtimox (*2*), some exhibiting a cure rate of zero. Parasite clearance rates for the Y, 12 SF, and Colombian strains of *T. cruzi* that had been treated with nifurtimox or with benznidazole are summarized in Table 6. Response to chemotherapy has been proposed as an important tool for classifying the strains of *T. cruzi* into different types, and in an investigation of the different responses of several strains of *T. cruzi* to allopurinol Avila et al. demonstrated that the most susceptible incorporated the drug several times faster than the insensitive strains (*18*).

In the present study, we were unable to detect any difference between the susceptibility of the three strains to MK-436. The index of cure was established on the basis of the results of the parasitological tests. However, the serological tests used (IIF and CML) remained positive for some mice that were considered to be parasitologically cured, which is consistent with the results of previous investigations (*2*) of drugs used in the chemotherapy of Chagas' disease. The proportion of mice that were positive in the CML test was higher for those infected with the Colombian strain. Analysis of the results of the treatment was therefore by no means straightforward. From 6 to 10 months after completion of treatment, focal infiltration of mononuclear cells was observed in several parasitologically cured mice, and this was more marked and frequent in those infected with the Colombian strain. In general, the Colombian strain and other type III strains of *T. cruzi* are more prone to produce myocardial lesions in the chronic phase of

the infection,[d] and this may have been responsible for our findings. In this respect it should be borne in mind that for the Colombian strain the mice with the most severe myocarditis also had positive CML tests. This could have been caused by residual parasitism in these mice that was not detected parasitologically; however, for mice infected with the Y and the 12 SF strains the myocardial infiltration was slight or absent, even for those with positive CML tests. The results of the histopathological study, showing the absence of lesions and parasites in mice with negative parasitological tests, may be strong evidence that they were cured.

The findings reported indicate that MK-436 exhibits a clear anti-trypanosomal effect *in vivo* and that it acts directly on intracellular *T. cruzi* parasites. However, the presence of ultrastructural lesions of non-parasitized cardiac fibres in treated mice should be borne in mind.

[d] ANDRADE, S. G. [*The murine model of chronic Chagas' myocardiopathy*]. Ph.D. thesis, Salvador-Bahia, 1985 (in Portuguese).

ACKNOWLEDGEMENTS

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RÉSUMÉ

ACTIVITÉ THÉRAPEUTIQUE DU MK-436 (NITRO-2,5 IMIDAZOLE) CONTRE *TRYPANOSOMA CRUZI* CHEZ LA SOURIS: ÉTUDE PARASITOLOGIQUE, SÉROLOGIQUE, HISTOPATHOLOGIQUE ET ULTRASTRUCTURALE

L'activité du MK-436 [(méthyl-1 nitro-5 imidazolyl-2)-3 hexahydro-3a,4,5,6,7,7a-benzisoxazole-1,2] a été testée chez la souris contre trois souches de *Trypanosoma cruzi* présentant des caractéristiques morphobiologiques et isoenzymatiques différentes: la souche Y (type I), la souche 12 SF (type II) et la souche colombienne (type III); dans des études précédentes, ces souches avaient montré différents degrés de sensibilité au nifurtimox et au benznidazole. Les souris ont été infestées avec 50×10^4 trypomastigotes sanguins et on leur a administré le MK-436 par gavage en deux prises quotidiennes de 250 mg/kg de poids corporel, soit pendant 10 jours, soit pendant 60 jours. On a étudié l'action intracellulaire directe du composé sur le parasite en observant en microscopie électronique le muscle cardiaque de souris sacrifiées 6 ou 24 heures après avoir reçu la première dose.

Tous les animaux traités ont été soumis à des tests de guérison parasitologique et à des épreuves sérologiques: immunofluorescence indirecte (IFI) et hémolyse en présence du complément. On a aussi procédé à l'examen histopathologique des souris sacrifiées pendant et après traitement.

Les résultats obtenus montrent que le MK-436 a un effet important sur la parasitémie, qui a disparu en 24 heures, que le traitement ait été appliqué au début ou vers la fin de la période d'infection aiguë. Les études ultrastructurales ont montré des altérations intracytoplasmiques graves chez le parasite. Les réactions sérologiques sont restées positives chez la plupart des souris traitées qui montraient une guérison parasitologique, notamment chez celles qui étaient infestées par la souche colombienne. Les taux de guérison, calculés à partir des résultats négatifs obtenus dans les épreuves parasitologiques, allaient de 72% à 100%, indépendamment de la souche et de la durée du traitement. Nos résultats montrent que le MK-436 a un effet nettement trypanosomicide *in vivo*, et qu'il a une action directe sur *T. cruzi* au niveau intracellulaire.

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