Mem. Inst. Oswaldo Cruz, Rio de Janeiro, Vol. *88* (1): 149-153, jan./mar. 1993

AN EXPERIMENTAL AND CLINICAL ASSAY WITH KETOCONAZOLE IN THE TREATMENT OF CHAGAS DISEASE

ZIGMAN BRENER; JOAQUIM ROMEU CANÇADO*; LUCIA MARIA DA CUNHA GALVÃO; ZÉLIA MARIA PROFETA DA LUZ; LENY DE SOUSA FILARDI; MARIA ELIZABETH SOARES PEREIRA; LUIZ MAURO T. SANTOS & CATARINA B. CANÇADO

Centro de Pesquisas René Rachou, FIOCRUZ, Caixa Postal 1743, 30190-002, Belo Horizonte, MG, Brasil
*Faculdade de Medicina, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil

Ketoconazole, an azole antifungic drug which is already in the market has also been demonstrated to be active against Trypanosoma cruzi *experimental infections. In this paper we confirmed the drug effect and investigated its range of activity against different* T. cruzi *strains naturally resistant or susceptible to both standard drugs Nifurtimox and Benznidazole used clinically in Chagas disease. Moreover, we have shown that the association of Ketoconazole plus Lovastatin (an inhibitor of sterol synthesis), which has an antiproliferative effect against* T. cruzi in vitro, *failed to enhance the suppressive effect of Ketoconazole displayed when administered alone to infected mice. Finally, administration in chronic chagasic patients of Ketoconazole at doses used in the treatment of deep mycosis also failed to induce cure as demonstrated by parasitological and serological tests. The strategy of identify and test drugs which are already in the market and fortuitously are active against* T. cruzi *has been discussed.*

Key words: *Trypanosoma cruzi* – Chagas disease – Ketoconazole

Chagas disease is endemic in most Latin America and causes at the chronic phase a severe myocardiopathy and pathological dilatations of the digestive tract. In the last three decades only two drugs, a nitrofuran (Nifurtimox) and a nitroimidazole (Benznidazole) had been submitted to clinical trials and then used in chagasic patients (Brener, 1984). Both drugs are potentially toxic nitroheterocyclic compounds which are administered in long term schedules and although inducing high rates of cure at the acute phase only a small percentage of the chronic cases are cured. Since the prospects of the introduction of new compounds by the pharmaceutical industry are poor, alternative strategies are being designed to identify, among drugs already available in the market for the treatment of other diseases, those that show activity against *Trypanosoma cruzi* and could be used clinically in Chagas disease (Avila & Avila, 1981; Gonzalez-Perdomo et al., 1990).

In this paper we used this approach and investigated further in animals and chronic chagasic patients the effects of Ketoconazole, a known azole antifungic drug which is now in the market and whose activity against *T. cruzi* has been previously described (Raether & Seidenhat, 1984; McCabe et al., 1983, 1987). We tested in mice its activity against different *T. cruzi* strains and also a possible synergistic effect of Ketoconazole associated with Lovastatin which is an inhibitor of hydroxymethylglutaryl-CoA reductase, a key enzyme in cholesterol biosynthesis. A synergistic effect of both drugs against *T. cruzi* in axenic culture and infected tissue culture has been recently reported by Urbina et al. (1991). Finally, we used Ketoconazole in a clinical trial on a group of chronic adult chagasic patients followed-up for eight months to over four years after treatment.

Received 8 July 1992.
Accepted 13 October 1992.

MATERIAL AND METHODS

Drug used – Cis-1-acetyl-4-(4-[2,4-dichlorophenyl)-2-1H-1-imidazolyl-methyl)-1,3-dioxolan-4-yl] methoxy] phenyl)-piperazine (Ketoconazole). [3-methyl-4-(5'-nitrofurfurylidene-amino)-tetrahydro-4H-thiazine-1,1-dioxide (Nifurtimox). 2-nitroimidazol (N-benzyl-2-nitro-1-imidazolacetamide (Benznidazole). [1S-[1 (R*),3, 7, 8 (2S*, 4S*),8a]] 1,2,3,7,8,8a-

hexahydro-3,7-dimethyl-8-[2(tetrahydro-4-hydroxy-6-oxo-2H-pyran- 2-yl) ethyl]-1-naphthalenyl methylbutanoate (Lovastatin).

T. cruzi strains – The following strains highly resistant to Nifurtimox and Benznidazole were used in experiments: *Noel,* isolated from an acute case; *Colombiana* (Federici et al., 1964); *VL-10* (Schlemper Jr, et al., 1983); *SC-28,* isolated from a marsupial *(Didelphis azarae); YuYu,* isolated from a naturally infected *Triatoma infestans.* The strains susceptible to both drugs were *Buriti* and *CL,* isolated from naturally infected *T. infestans.* The *Y* strain (Pereira da Silva & Nussenzweig, 1953) displays intermediary susceptibility to the drugs. More details on the natural resistance and susceptibility of the *T. cruzi* strains to both nitroheterocyclic derivatives can be found in a previous study by Filardi & Brener (1987).

Treatment of chronic chagasic patients – Eight adult chronic chagasic patients were treated and clinically followed-up by one of us (J.C.R.) in the Hospital das Clínicas, School of Medicine, University of Minas Gerais, after informed consent. They received Ketoconazole (3.1 to 8.7 mg/kg) by oral route for 51 to 96 days. More details on the treatment and patients are shown in Table III.

Treatment of infected mice – Groups of Swiss albino mice, 18-20g weight were inoculated intraperitoneally with either 1 x 10^4 or 2 x 10^4 blood forms of the different *T. cruzi* strains used in the experiments. The drugs were diluted with distilled water and given to mice by oral route through gavage. Different doses were administered in a daily portion and treatment started 24 hours after infection. In the experiments with different strains the drugs were given for 20 consecutive days. The cure rates were evaluated by hemocultures carried out 30 days after the end of the treatment. Untreated mice were used as controls. For the evaluation of the synergistic effect of the association Ketoconazole *plus* Lovastatin the number of blood forms was determined according to Brener (1962) at the peak of parasitemia, which occurs with the Y strain on the 7th day of infection. Mortality of the mice was daily recorded up to 20th day of infection.

Serological diagnostic tests – Sera were collected from the patients at the Hospital das Clínicas where the clinical follow-up was carried out. Aliquots of 2-3 ml were kept at –20 °C, the serological tests performed blind and the code disclosed afterwards. The complement-fixation test was performed according to Freitas & Almeida (1949). The indirect immunofluorescence test (IIF) was done using epimastigote stages yielded from LIT (Liver-infusion tryptose) medium and fluorescein conjugated anti-human IgG, according to Camargo & Takeda (1979). Indirect haemoagglutination test was carried out with the HAI antigen (Immunoserum, São Paulo).

Complement-mediated lysis – This test was performed according to Krettli et al. (1979) with small modifications. Briefly, blood forms collected from immunosuppressed mice or tissue culture trypomastigotes (6 x 10^6/ml) are incubated with fresh human serum as source of complement, at 37 °C for 45 min and then have their number determined in order to ascertain that the parasites are not lysed. Fifty microliters of the parasite suspension are added to 50 µl of the patient inactivated serum diluted at 1:2 and 1:4 and then the tubes are incubated at 37 °C, 30 min. Fifty microliters of complement are added to both tubes and the number of trypomastigotes determined in a haemocytometer; after incubation at 37 °C for 45 min the tubes are maintained in ice and the trypomastigotes again counted. The percentage of lysed parasites is calculated and tests in which lysis is > 20% are considered as positive.

Hemoculture in treated patients – The technique reported by Chiari et al. (1989) was used. Thirty mililiters of blood was collected, heparin (390IU) added and the material centrifuged for 10 min at 300 g at room temperature. Ten mililiters of LIT medium was added to the packed red blood cells which was centrifuged at 4 °C, 900 g for 30 min. The pellet was then ressuspended in 6 ml of LIT and divided into six tubes containing 3 ml of LIT medium. The tubes were kept at 26-28° and monthly examined microscopically for up to three months.

Hemoculture in infected mice – The animals were bled from the orbital sinus 30 days after the treatment and 0.2-0.4 ml of blood inoculated into tubes containing 5 ml of LIT medium. The tubes were kept at 28° and microscopically examined after 30 and 60 days.

RESULTS

Table I shows the results of the treatment with Ketoconazole (120 mg/kg, p.o., 20X), Benznidazole (100 mg/kg, p.o., 20X) and

TABLE I

Percentages of cure in group of mice inoculated with 1 x 10^4 blood forms of different *Trypanosoma cruzi* strains and treated for 20 consecutive days by oral route with Benznidazole (100 mg/kg), Nifurtimox (100 mg/kg) and Ketoconazole (120 mg/kg)

Strains	Benznidazole	Nifurtimox	Ketoconazole
Noel	3.4	3.4	42.8
Colombiana	6.6	0.0	7.6
VL-10	20.0	7.6	6.6
SC-28	3.5	0.0	80.0
YuYu	3.5	6.6	60.0
Buriti	100.0	100.0	45.4
CL	100.0	93.3	93.3
Y	50.0	66.6	100.0

Nifurtimox (100 mg/kg, p.o., 20X) of mice inoculated by intraperitoneal route with 10^4 blood forms of the different *T. cruzi* strains. A diversity of cure rate was observed in the groups of mice treated with Ketoconazole, as also occurs when Benznidazole and Nifurtimox are used instead. However, two out of the five strains resistant to the nitro derivatives were clearly also resistant to Ketoconazole as demonstrated by the very low percentages of cure (7.6 and 6.6%) observed with the latter compound (Table I). In relation to the strains *Buriti* and *CL*, highly susceptible to Nifurtimox and Benznidazole, the results with Ketoconazole agree with *CL* but only partially with *Buriti*. The results with the *Y* strain show that Ketoconazole is apparently more active than the nitro derivatives. Taken together the data indicate that Ketoconazole, as happens with other active derivatives, has a large range of activity that is contingent to the parasite sensitivity.

TABLE II

Parasitemia and mortality in groups of mice inoculated with 2 x 10^4 blood forms of the *Trypanosoma cruzi Y* strain and treated with Ketoconazole, Lovastatin and association of both drugs

Drug (mg/kg)	No. parasites/5 μl (7th day)	Mortality (20th day)
Ketoconazole (100)	0	0/6
Ketoconazole (40)	780	1/6
Ketoconazole (25)	14.670	1/5
Lovastatin (100)	64.333	6/6
Ketoconazole (40) Lovastatin (10)	689	1/5
Ketoconazole (25) Lovastatin (25)	15.480	4/5
Untreated controls	19.021	5/5

In Table II are shown results obtained in groups of mice inoculated with 2 x 10^4 blood forms from the *Y* strain and treated with Ketoconazole and Lovastatin alone as well as different associations of both drugs. A marked suppressive effect on the parasitemia was observed in the groups of mice treated daily with 100 and 40 mg/kg of Ketoconazole alone; at the doses of 25 mg/kg the drug was practivally unable to control the infection. Lovastatin at the dose of 100 mg/kg apparently increases the parasitism. The association of Lovastatin

TABLE III

Results of the treatment of chronic chagasic patients with Ketoconazole. Cure evaluation was performed by hemoculture, conventional serology and complement-mediated lysis

Patient	Treatment (days)	Dose (mg/kg)	Cure evaluation months after treatment	CoML[a] Serum dilution 1:2	CoML[a] Serum dilution 1:4	IIF	CFT	IHA	Hemoculture
S.R.P.	90	5.0	48	58	64	1:640	R[b]	1:64	Positive
M.L.G.M.	96	8.0	52	55	53	1:320	R	1:16	Positive
V.D.O.G.	96	8.3	36	94	96	1:640	R	1:64	Positive
H.M.C.	95	8.5	35	40	24	1:640	R	1:64	Positive
Z.M.P.S.	90	8.7	8	31	0	1:640	R	ND	Positive
N.M.S.	91	6.6	25	81	75	1:640	R	1:64	ND[c]
G.A.F.	73	4.5	20	47	67	1:640	R	1:64	ND
A.G.V.	102	8.8	60	ND		1:640	R	1:64	Positive

a: CoML (complement-mediated lysis), IIF (indirect immunofluorescence), CFT (complement fixation test), IHA (indirect hemoagglutination).
b: reactive.
c: not determined.

and Ketoconazole failed to enhance the suppressive effect induced by Ketoconazole "per se". The mortality rates closely parallels the results of the effects of the different treatment schedules on the parasitemia.

Table III displays data from the eight chronic patients treated with Ketoconazole. Six patients fail to be cured as demonstrated by the positivity of hemoculture carried out after treatment. In the remaining two cases in which the hemocultures were not performed the positivity of the serological tests, particularly the CoML test, strongly suggest the parasite persistence in the patients. No side-effects had been detected within the period of drug administration.

DISCUSSION

Only two drugs active against *T. cruzi,* Nifurtimox and Benznidazole, reached the pharmaceutical market in the last decades and have been used in chagasic patients. Both compounds share the inconveniences of nitro derivatives and although recommended for the treatment of acute cases they play a limited role in the specific therapy of chronic cases. This situation reflects the lack of interest of the pharmaceutical industry in the screening and development of new drugs for Chagas disease. As a consequence of this fact, it would be worthwhile to search for medicaments that, although designed for the treatment of diseases not related to Chagas disease, fortuitously exhibit activity against *T. cruzi* infections.

Allopurinol, for instance, an inhibitor of purine synthesis used for the treatment of gout in humans has shown to be active in *T. cruzi* infected mice (Avila & Avila, 1981). This drug failed to cure patients at the acute phase of Chagas disease treated with daily doses of 20 to 30 mg/kg (Lauria-Pires et al., 1988). However, more recently, Allopurinol has been considered as efficacious as Benznidazole and Nifurtimox in the treatment of groups of chronic chagasic patients in Argentine treated with 600 or 900 mg/day and evaluated by conventional serology and xenodiagnosis (Gallerano et al., 1990). Bacterial Topoisomerase II inhibitors (Ofloxacin and its commercial derivative Tarivid, nalidixic acid and Novobiocin) have been demonstrated to inhibit proliferation and differentiation of *T. cruzi* in axenic culture as well as in infected tissue culture (Gonzalez-Perdomo et al., 1990). No evidence of *in vivo* activity to the compounds has been so far provided.

A number of azole derivatives with a large spectrum of antifungal activity have been synthesized in the last years. They are used topically and also orally administered for the treatment of deep mycosis. Interestingly, some of these drugs such as Ketoconazole and Itraconazole which are in the market are also fairly active *in vivo* against *T. cruzi* experimental infections (McCabe et al., 1983, 1987). In this paper we expanded the experience with Ketoconazole by testing it in animals inoculated with several strains naturally resistant or susceptible to Nifurtimox and Benznidazole, the standard drugs used in the treatment of chagasic patients. Our results in animals inoculated with the *Y* strain cure agree with those reported by McCabe et al. (1987) who used the same strain in their experiment. At least two strains (*Colombiana* and *VL-10*), however, are naturally resistante to Ketoconazole according to our data.

The use of hemoculture as a criterion of cure in mice treated at the acute phase of *T. cruzi* infection was based on data from our laboratory showing a very strict correlation between this parasitological test and serology. In a group of 425 mice inoculated with different *T. cruzi* strains and treated with Nifurtimox or Benznidazole the correlation between hemoculture and indirect immunofluorescence (IIF) was of 92.9%; the percentage of mice with negative hemocultures and positive IIF was of 2.6%. In 203 untreated chronically infected mice only two presented negative hemocultures (Filardi & Brener, 1987).

In other series of experiences we investigated *in vivo* a presumable synergistic effect of an association between Ketoconazole and Lovastatin which is also in the market and is used to lower levels of cholesterol in humans. These experiences were based on a previous communication of Urbina et al. (1991) in which they demonstrated *in vitro* an antiproliferative effect of this association on *T. cruzi* epimastigotes grown in LIT medium and on amastigotes in tissue culture cells. In our experiments Lovastatin was unable to potentiate the suppressive effect of Ketoconazole on the parasitemia in mice inoculated with the *Y* strain. Intringuinly, administration to mice of Lovastatin alone enhances the parasitemia.

The treatment of the chronic chagasic patients with Ketoconazole for about three running months at doses usually used in the treat-

ment of deep mycosis was unable to induce parasitological cures as demonstrated by the positivity of hemocultures in five out of seven cases. The two remaining cases that could not be submitted to hemocultures are not in all likelihood cured as demonstrated by the presence in their sera of "lytic antibodies" detected by the complement-mediated lysis test. As previously reported "lytic antibodies" in Chagas disease are associated to resistance and indicate the presence of active ongoing *T. cruzi* infection (Krettli & Brener, 1982; Brener & Krettli, 1990). Moreover, this test has been used to evaluate treatment efficacy in Chagas disease and there is sound evidence that its persistent positivity indicates therapeutic failure (Krettli et al., 1982). Interestingly, these data concur with the finding reported by McCabe (1988) that Ketoconazole failed to cure chronic murine Chagas disease.

Experimental data in the chemotherapy of parasitic diseases are often misleading and do not correspond with the results in the human disease, the ultimate stage of the drug development process. The identification of compounds already in the market that have demonstrated to be active against *T. cruzi* may represent a by-pass to circumvent the extremely high costs demanded by drug development. The experience accumulated in the endemic area by different groups engaged on Chagas disease research allows that the whole process of experimental and clinical investigation be locally carried out with these drugs.

REFERENCES

AVILA, J. L. & AVILA, A., 1981. *Trypanosoma cruzi:* Allopurinol in the treatment of mice with experimental acute Chagas disease. *Exp. Parasitol., 51:* 204-208.

BRENER, Z., 1962, Therapeutic activity and criterion of cure on mice experimentally infected with *Trypanosoma cruzi. Rev. Inst. Med. trop. São Paulo, 4:* 389-396.

BRENER, Z., 1984. Recent advances in the chemotherapy of Chagas disease. *Mem. Inst. Oswaldo Cruz, 79* (suppl.): 149-156.

BRENER, Z. & KRETTLI, A. U., 1990. Immunology of Chagas disease, p. 247-261. In David J. Wyler, *Modern Parasite Biology. Cellular, Immunological and Molecular Aspects* W. H. Freeman and Company, New York.

CAMARGO, M. & TAKEDA, G. K. F., 1979. Diagnóstico de Laboratório. In p. 175-198. Z. Brener & Z. Andrade (eds) *Trypanosoma cruzi e Doença de Chagas,* Guanabara Koogan, Rio de Janeiro.

CHIARI, E.; DIAS, J. C. P.; LANA, M. & CHIARI, C. A., 1989. Hemocultures for the parasitological diagnosis of human chronic Chagas disease. *Rev. Soc. Bras. Med. Trop., 22:* 19-23.

FEDERICI, E. E.; ABELMAN, W. H. & NEVA, F. A., 1964. Chronic and progressive myocarditis and myositis in C3H mice infected with *Trypanosoma cruzi. Am. J. Trop. Med. Hyg., 13:* 272-280.

FILARDI, L. S. & BRENER, Z., 1987. Susceptibility and natural resistance to *Trypanosoma cruzi* strains to drugs used clinically in Chagas disease. *Trans. R. Soc. Trop. Med. Hyg., 81:* 755-759.

FREITAS, J. L. P. & ALMEIDA, J. O., 1949. Nova técnica de fixação de complemento para moléstia de Chagas. *O Hospital, 35:* 35-50.

GALLERANO, R. H.; MARR, J. & SOSA, R. R., 1990. Therapeutic efficacy of allopurinol in patients with chronic Chagas disease. *Am. J. Trop. Med. Hyg., 43:* 159-166.

GONZALEZ-PERDOMO, M.; CASTRO, S. L.; MEIRELLES, M. N. S. L. & GOLDENBERG, S., 1990. *Trypanosoma cruzi* proliferation and differentiation are blocked by Topoisomerase II inhibitors. *Antimicrob. Agents Chemother., 34:* 1707-1714.

KRETTLI, A. U. & BRENER, Z., 1982. Resistance against *Trypanosoma cruzi* associated to anti-living trypomastigotes antibodies. *J. Immunol., 128:* 2009-2012.

KRETTLI, A. U.; WEISZ-CARRINGTON, P. & NUSSENZWEIG, R. S., 1979. Membrane-bound antibodies to bloodstream *Trypanosoma cruzi* in mice: strain differences in susceptibility to complement-mediated lysis. *Clin. exp. Immunol., 37:* 416-423.

LAURIA-PIRES, L.; CASTRO, C. N.; EMANUEL, A. & PRATA, A., 1988. Ineficácia do allopurinol em pacientes na fase aguda da doença de Chagas. *Rev. Soc. Bras. Med. Trop., 21:* 79.

McCABE, R. E., 1988. Failure of Ketoconazole to cure chronic murine Chagas disease. *J. Infect. Dis., 158:* 1408-1409.

McCABE, R. E.; ARAÚJO, F. G. & REMINGTON, J. S., 1983. Ketoconazole protects against infection with *Trypanosoma cruzi* in a murine model. *Am. J. Trop. Med. Hyg., 32:* 960-962.

McCABE, R. E.; REMINGTON, J. S. & ARAÚJO, F. G., 1987. Ketoconazole promotes parasitological cure of mice infected with *Trypanosoma cruzi. Trans. R. Soc. Trop. Med. Hyg., 81:* 613-615.

PEREIRA DA SILVA, L. H. & NUSSENZWEIG, V., 1953. Sobre uma cepa de *Trypanosoma cruzi* altamente virulenta para o camundongo branco. *Folia Clin. et Biol., 20:* 191-208.

RAETHER, W. & SEIDENATH, H., 1984. Ketoconazole and other potent antimycotic azoles exhibit pronounced activity against *Trypanosoma cruzi, Plasmodium berghei* and *Entamoeba histolytica in vivo. Z. Parasitenkd., 70:* 135-138.

SCHLEMPER JR, B. R.; AVILLA, C. M.; COURA, J. R. & BRENER, Z., 1983. Course of infection and histopathological lesions in mice infected with seventeen *Trypanosoma cruzi* strains isolated from chronic patients. *Rev. Soc. Bras. Med. Trop., 16:* 23-30.

URBINA, J. A.; MALDONADO, R. A.; PAYARES, G.; MARCHAN, E.; LAZARDI, K.; AGUIRRE, T.; PIRAS, M. M.; PIRAS, R. & DE SOUZA, W., 1991. *In vitro* and *in vivo* synergism of binary and ternary combinations of ergosterol biosynthesis inhibitors as antiproliferative agent against *Trypanosoma cruzi:* therapeutic implications. *Mem. Inst. Oswaldo Cruz, 86* (suppl. I): 33-35.