# Cure of Short- and Long-Term Experimental Chagas' Disease Using D0870

Julio A. Urbina,* Gilberto Payares, Judith Molina, Cristina Sanoja, Andreína Liendo, Keyla Lazardi, Marta M. Piras, Romano Piras, Norma Perez, Patrick Wincker, John F. Ryley

Chagas' disease, a protozoan infection by the kinetoplastid *Trypanosoma cruzi*, constitutes a major public health problem in Latin America. With the use of mouse models of both short- and long-term forms of the disease, the efficacy of D0870, a bis-triazole derivative, was tested. D0870 was able to prevent death and induced parasitological cure in 70 to 90 percent of animals, in both the short- and long-term disease. In contrast, currently used drugs such as nifurtimox or ketoconazole prolonged survival but did not induce significant curing effects. D0870 may be useful in the treatment of human long-term Chagas' disease, a condition that is currently incurable.

Chagas' disease afflicts 16 million to 18 million persons in Latin America, causes one of the heaviest disease burdens of the region, and ranks third most prevalent at a global level among parasitic diseases, after malaria and schistosomiasis (*1*). Currently, no treatment is available for the long-term form of the disease, which is prevalent and leads in 30 to 40% of the cases to irreversible cardiac and gastrointestinal tract lesions (*2*). Nitrofurans such as nifurtimox (Bayer, recently discontinued) and nitroimidazoles such as benznidazole (Roche) have been used in short-term cases, but their efficacy varies between geographical areas, probably as a consequence of variation among parasite strains; moreover, both drugs have serious side effects, including anorexia, vomiting, peripheral polyneuropathy, and allergic dermopathy, which may be a consequence of oxidative or reductive damage in the host's tissues (*2*, *3*).

Sterol biosynthesis inhibitors (SBI) have been developed as chemotherapeutic agents for the treatment of fungal diseases and are often used in orally active forms (*4*). Their specific activity on fungal diseases is based on the depletion of essential endogenous sterols or the accumulation of toxic intermediates or both. *T. cruzi* is also sensitive to this class of inhibitor (*5*–*9*), but currently available SBIs, such as ketoconazole and itraconazole, are not powerful enough to eradicate the parasite from human patients or experimentally infected animals (*10*). The chemical (*R*,*S*)-2-(2,4-difluorophenyl)-1-(3-[(*Z*)-4-(2,2,3,3-tetrafluoropropoxy)styryl]-1,2,4-triazol-1-yl)-3-(1,2,4-triazol-1-yl)-propan-2-ol, also called as ICI 195,739, is a racemic compound with superior activity against systemic fungal infections in a variety of animal models and with a specific activity against *T. cruzi* in a murine model (*11*). On the basis of biochemical and ultrastructural studies, we concluded that ICI 195,739 has a dual mechanism of action against the parasite and involves inhibition of the sterol biosynthesis pathway at the level of cytochrome P450–dependent C14α-demethylase and a blockade of the cell cycle at cytokinesis (*7*, *12*). D0870, the *R*(+) enantiomer of ICI 195,739, is responsible for the specific antifungal activity (*13*), and this compound is currently being developed as a systemic antifungal agent (*14*).

In vitro work on both proliferative stages of the parasite showed that D0870 could also account for the anti–*T. cruzi* activity of ICI 195,739. Thus, against the epimastigote form, equivalent to the form present in the Reduviid vector and cultivable in axenic media (*15*), D0870 produced a dose-dependent effect on the growth rate and de novo synthesis of 4-desmethyl sterols, with median inhibitory concentrations ($IC_{50}$'s) of 100 and 50 nM, respectively, whereas for the *S*(−) enantiomer, the $IC_{50}$ for both growth inhibition and de novo sterol synthesis was 3 μM. Correspondingly, against the clinically relevant intracellular amastigote form, which was cultured in Vero cells (*5*, *6*, *9*), the minimal growth inhibitory concentration (m.i.c.) for D0870 was 10 nM, and no effects on the host cells were observed in the presence of drug concentrations 100 times greater. For the *S*(−) enantiomer, however, the m.i.c. was 300 nM, and deleterious effects on the host cells were already observed at 100 nM. In the presence of the m.i.c. of D0870, all the endogenous parasite sterols (ergosterol, 24-ethyl-cholesta-5,7,22-trien-3β-ol, their precursors in epimastigotes, and their 5,22-tetrahydro analogs in amastigotes) were replaced within 96 hours by 14α-methyl sterols such as lanosterol, 24-methylene-dihydrolanosterol, and 24-methyl-dihydrolanosterol (*16*). These results showed that the primary target of this compound, as well as of other azoles (*6*, *8*, *9*, *17*), is the parasite's sterol C14α-demethylase.

After we established that D0870 was responsible for the specific antiparasitic activity, this compound was used in subsequent in vivo studies. In a murine model of short-term disease (*6*, *8*, *9*), mice were infected with $10^5$ bloodstream trypomastigotes of the virulent Y strain, which led to a fulminant infection that killed all untreated animals in 21 days. Oral treatment was started 24 hours postinoculation (p.i.). The activity of D0870 was 30 to 50 times more potent than that of ketoconazole or nifurtimox in prolonging survival of infected animals (Table 1); moreover, D0870 given orally at 15 to 20 mg per kilogram of body weight per day (mg/kg per day) every other day (e.o.d.) for a total of 28 doses was able to protect 85 to 100% of the infected animals from death at the end of the observation periods (100 to 120 days). Animals treated with ketoconazole at 30 mg/kg per day given daily for a total of 43 doses or with suboptimal doses of D0870 began to die at 70 days p.i.; this delayed death was associated with the reappearance of circulating parasites at 60 days p.i. (*18*). No reappearance of circulating parasites occurred in animals treated with ≥15 mg/kg per day of D0870, which suggests that they had been cured by the drug treatment.

To verify the occurrence of parasitological cures of the short-term disease in surviving animals, we ran a battery of five independent tests, including hemoculture, xenodiagnosis, inoculation of homogenized organs into uninfected mice, hemoinoculation into baby mice, and detection of *T. cruzi* antibodies by immunoprecipitation of total epimastigote surface antigens labeled with $^{125}I$ (*6*, *7*, *9*, *19*). Additionally, we analyzed blood samples to detect specific *T. cruzi* DNA sequences by using a polymerase chain reaction (PCR) 'method, which has been recently shown to be the most sensitive technique available for parasite detection in patients with long-term disease (*20*). Greater than 60% of animals that received D0870 at 15 to 20 mg/kg per day were cured, whereas no cures were observed with currently available drugs (Table 1). Similar results were obtained if D0870 treatment

J. A. Urbina, A. Liendo, K. Lazardi, N. Perez, Laboratorio de Química Biológica, Centro de Bioquímica y Biofísica, Instituto Venezolano de Investigaciones Científicas, Apartado 21827, Caracas 1020A, Venezuela.
G. Payares, J. Molina, C. Sanoja, Departamento de Parasitología, Instituto de Zoología Tropical, Facultad de Ciencias, Universidad Central de Venezuela, Caracas 1040, Venezuela.
M. M. Piras and R. Piras, Unidad de Investigaciones, Centro Médico-Docente La Trinidad, Caracas 1080, Venezuela.
P. Wincker, Laboratoire 'Genome des Parasites', Faculte de Medecine, Université de Montpellier, 163, rue Auguste Broussonet, 34000 Montpellier, France.
J. F. Ryley, 2 Wych Lane, Adlington, Macclesfield, Cheshire SK10 4NB, UK.

*To whom correspondence should be addressed.

was started at 7 days p.i., when the infection was fully established and circulating parasites were found in all infected animals.

A murine model of long-term disease was also used in our studies; in this case, mice were infected with a less potent inoculum ($10^4$ bloodstream trypomastigotes) of the cardiotropic Bertoldo strain, which produced a slowly developing parasitemia that attained a peak at about 25 days p.i. but was effectively controlled by most infected animals; animals that survived this initial phase (~70%) experienced a slow deterioration of their general physical condition but survived for several months, although early sudden death also occurred. Treatment was started at 40 to 50 days p.i., at which time no circulating parasites were found. In this case, D0870 at 15 to 20 mg/kg per day given on alternate days for a total of 28 doses provided 90 to 100% protection from death with 80 to 90% parasitological cures, whereas conventional drugs given daily for a total of 43 doses had no significant effects on survival or number of cures when compared to controls (Table 2) (*21*).

The results suggest a parasitological cure of experimental long-term Chagas' disease. We think that the dual mechanism of action of D0870 against this parasite combined with its long lifetime in both rodents and humans (*14, 22*), which helps maintain high tissue concentrations of the drug for prolonged periods, could account for its antiparasitic activity. Our results indicate that D0870 could be useful in the treatment of human long-term Chagas' disease.

**Table 1.** Effect of D0870, ketoconazole, and nifurtimox on survival and parasitological cure in a murine model of short-term Chagas' disease. Groups that received daily treatment (or vehicle only for the control group) were treated for 28 consecutive days followed by a 7-day rest and another 15 days of treatment. Groups treated on alternate days received a total of 28 doses. For parasitological and serological tests, see (*6, 7, 9*). For DNA extraction and PCR of blood samples, see (*20*). The table summarizes the results of three independent experiments; blood PCR tests were carried out in one experiment. ND, not done. Shown is the number of affected individuals divided by the total number of individuals.

| Treatment | Total number of doses | Survival (105 days p.i.) | Negative parasitological, serological tests (105 days p.i.) | Negative blood PCR (105 days p.i.) |
|---|---|---|---|---|
| None (control) | 0 | 0/27 | 0/27 | 0/9 |
| Nifurtimox (50 mg/kg/day, daily) | 43 | 6/10 | 0/10 | n.d. |
| Ketoconazole (30 mg/kg/day, daily) | 43 | 13/27 | 1/27 | 1/9 |
| D0870 (1 mg/kg/day, daily) | 43 | 6/8 | 0/8 | ND |
| D0870 (1 mg/kg/day, e.o.d.) | 28 | 6/8 | 1/8 | ND |
| D0870 (5 mg/kg/day, daily) | 43 | 18/27 | 6/27 | 4/9 |
| D0870 (5 mg/kg/day, e.o.d.) | 28 | 8/8 | 1/8 | ND |
| D0870 (10 mg/kg/day, daily) | 43 | 24/28 | 11/28 | 3/10 |
| D0870 (10 mg/kg/day, e.o.d.) | 28 | 24/28 | 16/28 | 4/10 |
| D0870 (15 mg/kg/day, e.o.d.) | 28 | 17/20 | 12/20 | 7/10 |
| D0870 (20 mg/kg/day, e.o.d.) | 28 | 20/20 | 13/20 | 9/10 |
| D0870 (20 mg/kg/day, e.o.d.) treatment started 7 days p.i. | 28 | 10/10 | 6/10 | 6/10 |

**Table 2.** Effect of D0870, ketoconazole, and nifurtimox on survival and parasitological cure in a murine model of long-term Chagas' disease. Groups that received daily treatment (or vehicle only for the control group) were treated for 28 days followed by a 7-day rest and another 15 days of treatment. Groups treated on alternate days received a total of 28 doses. For parasitological and serological tests, see (*6, 7, 9*). For DNA extraction and PCR of blood samples, see (*20*). The table summarizes the results of two independent experiments. ND, not done. Shown is the number of affected individuals divided by the total number of individuals.

| Treatment | Total number of doses | Survival (145 days p.i.) | Negative parasitological, serological tests (145 days p.i.) | Negative blood PCR (145 days p.i.) |
|---|---|---|---|---|
| None (control) | 0 | 10/20 | 3/20 | 6/20 |
| Nifurtimox (50 mg/kg/day, daily) | 43 | 8/9 | 1/9 | ND |
| Ketoconazole (30 mg/kg/day, daily) | 43 | 14/19 | 3/19 | 3/19 |
| D0870 (10 mg/kg/day, daily) | 43 | 17/20 | 13/20 | 17/20 |
| D0870 (10 mg/kg/day, e.o.d.) | 28 | 14/20 | 12/20 | 12/20 |
| D0870 (15 mg/kg/day, e.o.d.) | 28 | 17/20 | 16/20 | 17/20 |
| D0870 (20 mg/kg/day, e.o.d.) | 28 | 20/20 | 19/20 | 18/20 |

## REFERENCES AND NOTES


1. World Bank, *World Development Report 1993: Investing in Health* (Oxford Univ. Press, Oxford, 1993).
2. A. Rassi and A. O. Luquetti, in *Chagas' Disease* (*American* Trypanosomiasis): Its Impact on Transfusion and Clinical Medicine, S. Wendel, Z. Brener, M. E. Camargo, A. Rassi, Eds. (*ISBT* BRAZIL'92, São Paulo, Brazil, 1992), p. 237.
3. S. L. de Castro, *Acta Trop.* **53**, 83 (1993).
4. H. Vanden Bossche, in *Modern Selective Fungicides: Properties, Applications, Mechanism of Action*, H. Lyr, Ed. (Gustav Fisher Verlag, Jena, Germany, 1995), p. 431; Vanden Bossche and P. Marichal, in *Recent Progress in Antifungal Chemotherapy*, H. Yamaguchi, G. S. Kobayashi, H. Takahashi, Eds. (Marcel Dekker, New York, 1992), p. 25.
5. J. A. Urbina, K. Lazardi, T. Aguirre, M. M. Piras, R. Piras, *Antimicrob. Agents Chemother.* **32**, 1237 (1988).
6. J. A. Urbina *et al.*, *ibid.* **37**, 580 (1993).
7. R. A. Máldonado, J. Molina, G. Payares, J. A. Urbina, *ibid.*, p. 1353.
8. J. A. Urbina, J. Vivas, G. Visbal, L. M. Contreras, *Mol. Biochem. Parasitol.* **73**, 199 (1995).
9. J. A. Urbina *et al.*, *Chemotherapy* **42**, 294 (1996).
10. R. E. McCabe, *J. Infect. Dis.* **158**, 1408 (1988); A. A. B. Moreira *et al.*, *Rev. Inst. Med. Trop. Sao Paulo* **34**, 177 (1992); Z. Brener *et al.*, *Mem. Inst. Oswaldo Cruz Rio J.* **88**, 149 (1993).
11. J. F. Ryley, S. McGregor, R. G. Wilson, *Ann. N.Y. Acad. Sci.* **544**, 310 (1988).
12. J. A. Urbina, K. Lazardi, T. Aguirre, M. M. Piras, R. Piras, *Antimicrob. Agents Chemother.* **35**, 730 (1991); K. Lazardi, J. A. Urbina, W. DeSouza, *ibid.*, p. 736.
13. H. Yamada *et al.*, *ibid.* **37**, 2412 (1993).
14. K. V. Clemons, L. H. Hanson, D. A. Stevens, *ibid.*, p. 1177; K. V. Clemons and D. A. Stevens, *J. Med. Vet. Mycol.* **32**, 323 (1994); B. A. Atkinson, R. Bocanegra, A. L. Colombo, J. R. Graybill, *Antimicrob. Agents Chemother.* **38**, 1604 (1994); K. V. Clemons and D. A. Stevens, *ibid.* **39**, 778 (1995); N. C. Karyotakis, M. C. Dignani, R. Hachem, E. J. Anaissie, *ibid.*, p. 571; H. M. Wardle, D. Law, C. B. Moore, C. Mason, D. W. Denning, *ibid.*, p. 868.
15. A. De Maio and J. A. Urbina, *Acta Cient. Venez.* **35**, 136 (1984).
16. Neutral lipids were extracted, purified, and analyzed by capillary gas-liquid chromatography coupled to mass spectrometry as described (*6, 8, 9*).
17. L. J. Goad, R. L. Berens, J. J. Marr, D. H. Beach, G. G. Holz Jr., *Mol. Biochem. Parasitol.* **32**, 179 (1989);

G. Larralde, J. Vivas, J. A. Urbina, *Acta Cient. Venez.* **39**, 140 (1988).
18. Statistical analysis of the survival curves, by use of both the log rank (Mantel-Cox) and Peto-Peto-Wilcoxon tests, indicated a significant ($P < 0.0001$) difference between the control (untreated) animals and all those that received the drug treatments, as well as between those that received D0870 at ≥15 mg/kg per day e.o.d. and the group treated with ketoconazole at 30 mg/kg per day daily. Survival analysis was carried out on the StatView program, version 4.5, run on a Power Macintosh 7100/66 computer.
19. We carried out hemocultures by inoculating 2 ml of liver infusion medium with 0.4 ml of blood obtained from experimental mice by cardiac puncture; microscopic examination of the cultures for the presence of proliferative epimastigote forms was done weekly for 4 weeks. Surviving animals were killed, and organs (spleen, liver, heart, and bone marrow) were minced individually in 1 ml of sterile, phosphate-buffered saline with 10 mM D-glucose; 0.4 ml of the suspension was inoculated in juvenile animals (15 to 20 g). Hemoinoculation (50 μl of blood diluted to 100 μl with sterile, phosphate-buffered saline) was done subcutaneously in 10- to 12-day-old mice. Xenodiagnosis was done with 10 second-stage *Rodnius prolixus* nymphs per mouse; after 2 weeks, the feces were analyzed for *T. cruzi* metacyclic forms, and the exam was repeated weekly thereafter for 1 month. The presence of circulating *T. cruzi* antibodies was detected by immunoprecipitation of $^{125}$I-labeled total epimastigote surface antigen antigens with experimental sera in the presence of protein A, followed by analysis of the precipitate by SDS–polyacrylamide gel electrophoresis.
20. C. Britto, M. A. Cardoso, P. Wincker, C. M. Morel, *Mem. Inst. Oswaldo Cruz Rio J.* **88**, 171 (1993); P. Wincker *et al.*, *Am. J. Trop. Med. Hyg.* **51**, 771 (1994). The *T. cruzi*–specific primers used in the tests were 5′-AAATAATGTACGGG(T/G)GAGATGCATGA-3′ and 5′-GGTTCGATTGGGGTTGGTGTAATATA-3′.
21. Statistical analysis of the survival curves, by use of both the log rank (Mantel-Cox) and Peto-Peto-Wilcoxon tests, indicated no significant differences between the control (untreated) animals and those that received ketoconazole at 30 mg/kg per day daily or D0870 at 10 mg/kg per day e.o.d., whereas there were significant differences between these groups and those receiving D0870 at 10 mg/kg per day daily ($P = 0.05$) or ≥15 mg/kg/day e.o.d. ($P = 0.005$).
22. S. De Wit, E. O'Doherty, R. P. Smith, R. Yates, N. Clumeck, *Intersci. Congr. Antimicrob. Agents Chemother. Abstracts* **35**, F97 (1995).
23. This work was supported by the UN Development Programme–World Bank–World Health Organization Programme for Research and Training in Tropical Diseases (grant 930161) and the National Research Council of Venezuela (Consejo Nacional de Investigaciones Cientificas y Técnicas, grant RP-IV-110034). We acknowledge technical assistance by R. Lira and G. Visbal. We dedicate this paper to the memory of Jose Witremundo Torrealba.

8 April 1996; accepted 12 June 1996

# Auditory Neurophysiologic Responses and Discrimination Deficits in Children with Learning Problems

Nina Kraus,* Therese J. McGee, Thomas D. Carrell, Steven G. Zecker, Trent G. Nicol, Dawn B. Koch

**Children with learning problems often cannot discriminate rapid acoustic changes that occur in speech. In this study of normal children and children with learning problems, impaired behavioral discrimination of a rapid speech change (/dɑ/versus/gɑ/) was correlated with diminished magnitude of an electrophysiologic measure that is not dependent on attention or a voluntary response. The ability of children with learning problems to discriminate another rapid speech change (/bɑ/versus/wɑ/) also was reflected in the neurophysiology. These results indicate that some children's discrimination deficits originate in the auditory pathway before conscious perception and have implications for differential diagnosis and targeted therapeutic strategies for children with learning disabilities and attention disorders.**

Learning and attention problems occur in many children, often concurrently (*1*). These disorders frequently involve an inability to process complex auditory information that occurs, for example, in speech. In fact, a large subset of children with such disorders cannot process complex auditory signals, even at the most elemental level (*2*, *3*).

A comprehensive study is under way to examine the relation among psychophysical speech discrimination abilities, standardized measures of learning and academic achievement, and neurophysiology in a large population of both normal children and children with learning problems. One aim is to determine whether children with certain auditory processing problems have difficulties that originate from abnormalities in the neurophysiologic encoding of acoustic differences in speech (which occurs after peripheral sensory encoding and before conscious perception) or whether the problems arise from some higher level processing deficit (which may involve, for example, linguistic or cognitive abilities) (*4*). Such information would aid in the diagnosis and treatment of these children, whose learning problems have been difficult to define or categorize.

An important aspect of this work is to establish a neurophysiologic correlate of behavioral discrimination. Fortunately, there is a neurophysiologic response that occurs in response to small (as well as large) acoustic changes in both simple and complex stimuli (*5*). This response, termed the mismatch negativity (MMN), provides an index of the neurophysiologic representation of acoustic contrasts and thus provides a tool for exploring the processing of acoustic differences that underlie speech perception.

The MMN originates in the auditory thalamocortical pathway (*6*, *7*) and demonstrates learning-associated plasticity (*8*). It is elicited by a physically deviant stimulus occurring in a series of homogeneous stimuli. The response can be elicited in a passive paradigm in which attention or behavioral responses are not required (*9*). It has been obtained during sleep in infants and adults and during wakefulness, sleep, and barbiturate anesthesia in animal models (*10*). From a developmental standpoint, the MMN is robust in children and appears to be mature by school age (*11*, *12*). Thus, the MMN reflects with considerable precision the discrimination of acoustic change and can be used to determine which aspects of the acoustic signal are differentiated neurophysiologically and, ultimately, which neuronal pathways are impaired (*7*, *13*).

In this experiment, behavioral discrimination abilities and MMN responses were evaluated in a group of normal children ($n = 90$) and in a group of children with learning problems ($n = 91$). The normal group consisted of children ages 6 to 15 years with no history of learning or attention problems (based on a detailed parent questionnaire) and scores within normal limits (including no discrepancy between ability and achievement) on a psychoeducational test battery (*14*). The group with learning problems consisted of children in the same age range who had been diagnosed clinically as having a learning disability (LD children), attention deficit disorder (ADD children), or both; in some cases, they had scores that were not within the normal limits on two or more of the tests in the psychoeducational test battery and a history of learning or attention difficulties (suspected LD). All children had normal intelligence (scores >85 on the Brief Cognitive Scale) (*14*). The normal group differed significantly from the group with

N. Kraus, Communication Sciences and Disorders, Northwestern University; and Departments of Neurobiology and Physiology, and Otolaryngology, Northwestern University, Evanston, IL 60208, USA.
T. J. McGee, S. G. Zecker, T. G. Nicol, D. B. Koch, Communication Sciences and Disorders, Northwestern University, Evanston, IL 60208, USA.
T. D. Carrell, Special Education and Communication Disorders, University of Nebraska, Lincoln, NE 68583, USA.
*To whom correspondence should be addressed. E-mail: nkraus@nwu.edu