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Hydroxymethylnitrofurazone Is Active in a Murine Model of Chagas' Disease▽

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The addition of a hydroxymethyl group to the antimicrobial drug nitrofurazone generated hydroxymethylnitrofurazone (NFOH), which had reduced toxicity when its activity against *Trypanosoma cruzi* was tested in a murine model of Chagas' disease. Four groups of 12 Swiss female mice each received 150 mg of body weight/kg/day of NFOH, 150 mg/kg/day of nitrofurazone (parental compound), 60 mg/kg/day of benznidazole (BZL), or the solvent as a placebo. Treatments were administered orally once a day 6 days a week until the completion of 60 doses. NFOH was as effective as BZL in keeping direct parasitemia at undetectable levels, and PCR results were negative. No histopathological lesions were seen 180 days after completion of the treatments, a time when the levels of anti-*T. cruzi* antibodies were very low in mice treated with either NFOH or BZL. Nitrofurazone was highly toxic, which led to an overall rate of mortality of 75% and necessitated interruption of the treatment. In contrast, the group treated with its hydroxymethyl derivative, NFOH, displayed the lowest mortality (16%), followed by the BZL (33%) and placebo (66%) groups. The findings of histopathological studies were consistent with these results, with the placebo group showing the most severe parasite infiltrates in skeletal muscle and heart tissue and the NFOH group showing the lowest. The present evidence suggests that NFOH is a promising anti-*T. cruzi* agent.

In 1909, Carlos Chagas described the protozoan parasite *Trypanosoma cruzi* as the causative agent of a widespread disease in Brazil that involved cardiac alterations and megaorgans. The parasite and its insect vectors were described in the original paper on the disease (7), and the parasite was soon confirmed to be present in patients throughout Latin America. Recent reports estimate the number of infected people to be between 9.8 million and 15 million in the Americas (29), with 28 million more being at risk of infection (26). In this scenario, congenital transmission of infection is a major problem in urban areas, along with risks from blood transfusion and migration of infected patients to areas of nonendemicity (28). In spite of the reduction in the number of new infections and the increased knowledge about the parasite, treatment of Chagas' disease relies on two drugs developed during the early 1970s: benznidazole (BNZ; Radanil, Roche) and nifurtimox (NFX; Bayer) (35). Both drugs are generally well tolerated by children but cause many undesirable side effects in adults and are not effective during the chronic phase of the disease. A well-tolerated, safe, and therapeutically efficient drug is not yet available. Several attempts have been made to create new drugs with more specificity toward the parasite and less toxicity for the mammalian host. Such attempts included inhibitors of cruzain (13, 14), C-14α-demethylase (5, 10, 32), and chemical modifications of molecules with known trypanocidal activity, such as aromatic nitroheterocyclic compounds (1, 2). Nitrofurazone is an antimicrobial drug that is effective against Gram-positive and Gram-negative bacteria but that also presents with anti-*T. cruzi* activity. In order to study new derivatives of nitrofurazone, the intermediate hydroxymethylnitrofurazone (NFOH) was found to be four times less mutagenic than the parental nitrofurazone compound in Ames tests (15). Moreover, NFOH's lethal dose is higher than 2,000 mg/kg of body weight (24). *In vitro* assays demonstrated that NFOH was more effective than benznidazole and nitrofurazone against trypomastigotes and amastigotes. NFOH was also shown to interfere with the steps of RNA transcription, suggesting that NFOH may affect the *trans*-splicing reaction (3). We tested the therapeutic effect of NFOH on an animal model of *T. cruzi* infection. This work represents a step forward in the study of NFOH, since reports previously published in the literature indicated successful *in vitro* test results with this promising anti-*T. cruzi* agent.

MATERIALS AND METHODS

Animals and parasites. Outbred female Swiss mice ($n = 48$) with an average weight of 20 g, bred in our facilities, were infected with 10^3 *T. cruzi* blood trypomastigotes by intraperitoneal injection. The strain of choice was Tulahuen, because it is susceptible to benznidazole treatment, displays a reproducible parasitemia curve, and is available in our laboratory. Breeding, maintenance, and experimentation with mice followed established guidelines (33). Bleedings for the collection of samples for determination of the level of parasitemia and serology were carried out by cutting 1 mm off the tail tip. Bleedings for the collection of samples for PCR were carried out while the mice were under anesthesia, and two blood samples were collected from the same mouse and pooled. The bleedings were done 7 days apart to allow the mice to recover. The animals were euthanized after the last bleeding for the collection of samples for PCR and serology and tissue sampling were carried out. All experiments with animals were approved by the Animal Ethics Committee of the Universidad Nacional de Salta.

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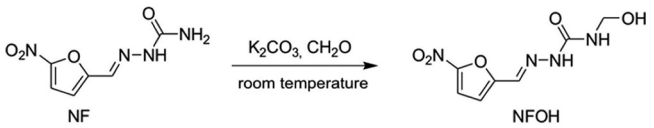

FIG. 1. Chemical structure of NFOH.

Experimental design. A completely randomized design with four groups of 12 infected animals each was set according to the following treatments: BZL at 60 mg/kg/day, NFOH at 150 mg/kg/day, NF at 150 mg/kg/day, and placebo. Treatments started at 5 days postinfection and were administered orally in a suspension of 9% NaCl–5% Tween 80 (placebo) once a day 6 days per week until the completion of 60 doses. The doses of NF and its derivative, NFOH, were established on the basis of their molecular weights, which are similar. The dose of BZL was selected on the basis of previous empirically determined doses, which displayed a low level of toxicity and efficacy in mice (12).

A second experiment with four groups of five healthy, uninfected animals each was set up to determine the levels of the enzyme serum glutamic oxaloacetic transaminase (SGOT) as a measure of liver alterations in response to long-term treatment with anti-*T. cruzi* agents. These mice received the same treatments as the infected groups. The NF group received only 45 mg/kg/day due to the high rate of mortality observed with the infected animals (16), whereas the BZL, NFOH, and placebo doses remained the same as those for the infected animals. SGOT levels were also measured in infected mice.

Drugs. Benznidazole (Radanil) was bought from Roche. 5-Nitro-2-furaldehyde *N*-(hydroxymethyl)-semicarbazone (NFOH) was synthesized from 5-nitro-2-fufurylidenesemicarbazone (NF), as described by Chung et al. in 2003 (9) (Fig. 1).

Parasitemia. Fresh blood (10 μl) was examined under a Zeiss optical microscope at ×400 magnification at 2- to 4-day intervals from days 5 to 46 postinfection. The number of parasites in 100 fields was counted.

Serological studies. Serum samples were taken at days 45 and 180 after treatment (105 and 240 days postinfection, respectively) and analyzed by enzyme-linked immunosorbent assay (ELISA) in 96-well plates. Briefly, the plates were covered with an homogenate of *T. cruzi* soluble proteins, and antibodies against *T. cruzi* in the sera (dilution 1:100) were detected with anti-mouse IgG (Sigma) at a 1:2,500 dilution and 1:16,000 avidin-biotin-peroxidase conjugate (Sigma). Reactions were revealed with SigmaFAST *o*-phenylenediamine tablets, and the optical density (OD) values at 490 nm were measured on a Labmet spectrophotometer. ELISAs were carried out twice with duplicate serum samples; therefore, each point in Fig. 2 to 4 represents the mean value of four replicates. The cutoff point was set from the mean of the negative controls plus 0.1 OD unit.

PCR. Blood samples were taken at 30 and 180 days after treatment (90 and 240 days postinfection, respectively). A volume of 700 μl was mixed with 1,400 μl guanidine buffer, and the mixture was boiled 24 h later to avoid clot formation. DNA was extracted using the phenol-chloroform method (4, 27) in a UV light-irradiated chamber under strict decontamination conditions. The amplification mixture was prepared in a different UV light-irradiated chamber and contained 3 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 0.1 μM primers 121 (5′-AAA TAA TGT ACG GG(T/G) GAG ATG CAT GA-3′) and 122 (5′-GGT TCG ATT GGG GTT GGT GTA ATA TA-3′), 1.65 U GoTaq polymerase (Promega), and 10 μl DNA. The amplification reaction was carried out in a thermal cycler (M. J. Research, Watertown, MA), using the following conditions: hot start (initial denaturation) at 94°C, followed by two cycles of 1 min at 98°C and 2 min at 64°C and then 33 amplification cycles (1 min at 94°C and 2 min at 64°C) and the final elongation (1 min at 72°C and 2 min at 25°C). The 330-bp amplicons were separated electrophoretically on 2% agarose gels, stained with ethidium bromide, and photographed with a Kodak EDAS system.

PCR controls. For the collection and extraction procedures, the negative control consisted of blood from a noninfected mouse. Positive-control samples were taken from infected mice with high levels of parasitemia. Both sets of control samples were collected along with the experimental samples. The positive control for the amplification procedure was purified DNA from a *T. cruzi* Tulahuen strain culture. A blank tube with water was included to rule out contamination in the PCR mixtures. Each run included a maximum of five duplicate samples, the controls, and a blank tube.

Histopathology. Samples of heart, skeletal muscle, intestine, and urinary bladder tissues were taken and stored in 10% formaldehyde, followed by dehydration in alcohol solutions and xylol and embedment in paraffin. Every organ was embedded separately in paraffin and cut in a microtome set at 6 μm. Slides were stained with hematoxylin-eosin and observed under a Zeiss microscope at ×250 magnification. Photographs were taken at ×400 magnification with an Olympus X-785 digital camera coupled to the microscope.

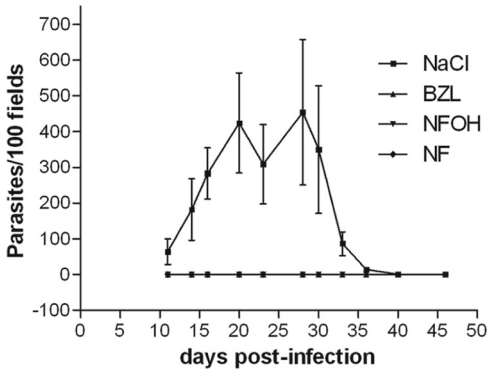

FIG. 2. Parasitemia curves for mice treated with BZL, NFOH, NF, and placebo.

Toxicity assays. The activity of SGOT is highly representative of liver damage (18). SGOT reacts with L-aspartate and α-ketoglutarate to ultimately form glutamate and pyruvate. Pyruvate reacts with 2,4-dinitrophenylhydracine, producing a colored compound that can be detected at 505 nm and whose presence is a measure of SGOT activity. SGOT determinations were carried out with a commercial kit (Wiener Lab, Rosario, Argentina). Briefly, 50 μl of serum was added to SGOT substrate (100 mM L-aspartate, 2 mM α-ketoglutarate, phosphate buffer, pH 7.4), and the mixture was incubated for 30 min at 37°C, followed by an incubation for 10 min at 37°C with 2,4-dinitrophenylhydracine (1 mM in 1 M chlorhydric acid). The reaction was stopped with the addition of 4 M NaOH and incubation for 5 min at room temperature. The absorbance was measured in a spectrophotometer at 505 nm. The concentration of pyruvate was determined by extrapolation from a calibration curve, which was obtained by performing the reaction with different concentrations of a known standard provided with the kit.

RESULTS

Parasitemia. During the first 2 months postinfection, the level of circulating parasites in each mouse was monitored by direct microscopic observation. Trypomastigotes were detectable in the blood of mice not treated with drug but were absent in mice treated with BZL, NFOH, or NF throughout the study (Fig. 2). Parasitemia became undetectable at 40 days postinfection in the placebo group. However, after 2 weeks of treatment, mice that received NF showed signs of toxicity (erected hair, dehydration, and inactivity). Therefore, the administration of NF stopped. Except for three survivors, all mice treated with NF died at a later stage. Toxicity was not observed either in BZL-treated or in NFOH-treated mice.

Serological studies. BZL-, NFOH-, and NF-treated mice maintained low levels of antibodies 45 days after completion of the treatments. All three drugs kept the serological titers to low levels, although there was one mouse treated with BZL and one treated with NFOH that displayed high OD values (Fig. 3A). However, on day 180 posttreatment (240 days postinfection), 50% of the BZL-treated mice and 30% of the NFOH-treated mice were negative. The differences between the BZL- or NFOH-treated mice and the negative controls were not statistically significant. Mice that received placebo showed high levels of antibodies, and one out of three treated with NF was clearly positive at 240 days postinfection, although parasitemia was undetectable. NF-treated mice had higher antibody levels at 240 days posttreatment (Fig. 3B). The negative

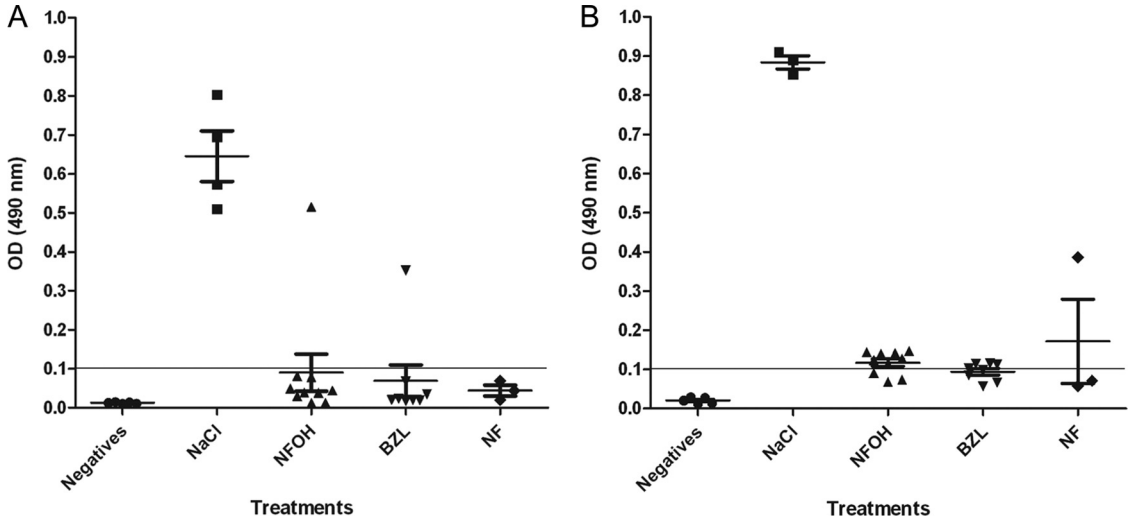

FIG. 3. Antibody levels after the completion of treatments with BZL, NFOH, NF, or placebo. NaCl corresponds to mice that received placebo. Samples from the same negative controls (healthy untreated mice) were used in every ELISA. The mean OD of each group is shown with a horizontal line, and the cutoff is the continuous line from the *y* axis at 0.1 OD. (A) Titers at 45 days after treatment (105 days postinfection); (B) titers at 180 days after treatment (240 days postinfection).

detection of antibodies in the NF group at 45 days posttreatment could be due to immunosuppression, a side effect of the toxicity.

PCR and mortality. Table 1 summarizes the results of PCR and the rates of mortality at 30 and 180 days posttreatment. Mice that received placebo showed a high rate of mortality due to *T. cruzi* infection (66%), although it was not as high as the rate of mortality due to NF toxicity (75%). Most NF-treated mice (67%) were PCR positive at 30 days posttreatment, but all were negative at 180 days posttreatment, whereas 75% of the placebo group remained positive at both times. In contrast, NFOH induced the lowest level of mortality of all treatments (16%), with only 10% of mice having positive PCR results at 30 days. After 180 days posttreatment, all mice treated with NFOH were PCR negative. BZL was effective in reducing the parasite load, as shown by the negative PCR results at 30 and 180 days posttreatment. The rate of mortality in the group treated with BZL was low (33%), although it was double that in the NFOH-treated group.

The log-rank test (Mantel-Cox test; GraphPad Prism software, version 5.0) showed an overall statistically significant difference between survival curves ($P = 0.0002$), in particular, that between the placebo and NFOH groups ($P = 0.0003$) and that between the placebo and BZL groups ($P = 0.0011$). However, the test indicated that there is no statistically significant difference between the NFOH and BZL groups in terms of the survival curves. Figure 4 illustrates these results.

Histopathology. Tissue sections were taken in the late chronic stage of infection. No significant alterations in heart, urinary bladder, or intestinal tissue were found. The skeletal muscle tissue of mice that received placebo showed slight lymphomonocytic infiltrates surrounding muscle fibers (Fig. 5A, asterisk) and calcification (Fig. 5A, arrow). Nuclear dysplasia, a condition characterized by the migration of nuclei from the surface to the center of the muscle cells, was also detected in this group (Fig. 5B). Healthy tissue is shown in Fig. 5C. Lymphomonocytic infiltrates, calcification, and nuclear dysplasia were suppressed in NFOH-treated mice and also in the survivors of the BZL and NF treatments. Mice that received BZL or NFOH displayed liver sections with scattered focal lymphomonocytic infiltrates in sinusoid capillaries and interlobular spaces. Figure 6 shows liver sections from an untreated animal without infection (Fig. 6A) and a lymphomono-

TABLE 1. Mortality and PCR results at 30 and 180 days posttreatment

Treatment	% mortality (no. dead/total no.)	% PCR positive (no. positive/total no.) at posttreatment day:	
		30	180
NaCl	66.6 (8/12)	75 (3/4)	75 (3/4)
BZL	33.3 (4/12)	0 (0/8)	0 (0/8)
NF	75 (9/12)	66.6 (2/3)	0 (0/3)
NFOH	16.6 (2/12)	10 (1/10)	0 (0/10)

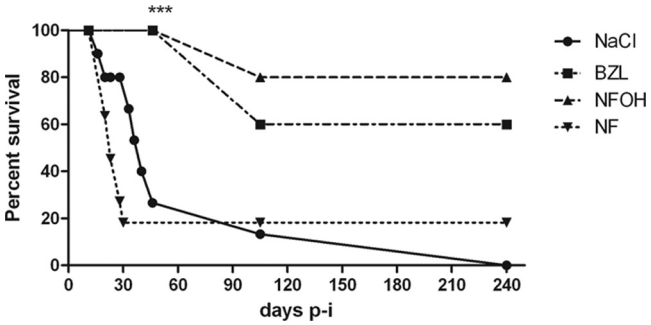

FIG. 4. Survival curves for infected and treated mice. Placebo- and NF-treated mice showed higher rates of mortality than mice for that received BZL or NFOH. Asterisks show statistically significant differences between these two sets of curves. p-i, postinfection.

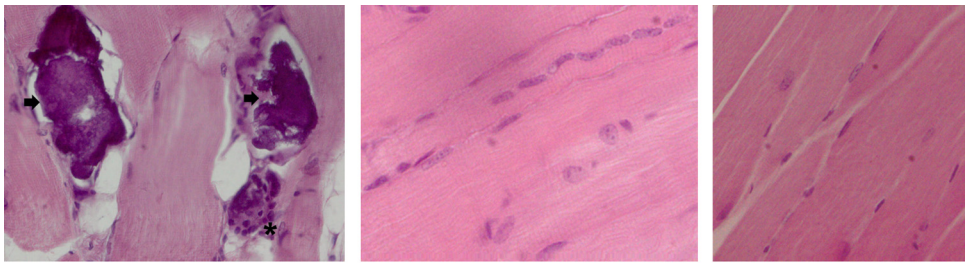

FIG. 5. Comparison of skeletal muscle of healthy mice and infected, untreated mice. (A) Lymphomonocytic infiltration (asterisk) and calcification (arrow) in infected, placebo-treated mice; (B) nuclear dysplasia in infected, placebo-treated mice; (C) muscle fibers of healthy mice. Hematoxylin-eosin staining. Magnification, ×400.

cytic infiltrate of a mouse infected with *T. cruzi* (Fig. 6B). NFOH-treated mice displayed low degrees of inflammatory infiltrates, similar to those found in the placebo group, in contrast to BZL, which induced the highest degree of liver inflammation. However, these differences were not statistically significant.

Toxicity assays. Measurements in the toxicity assay were taken at day 30 to allow a longer period of drug intake, given that at day 15 the infected mice did not show any difference in the activity of SGOT. Both groups were also tested for SGOT activity 1 week after the completion of the treatments. Normal values were obtained from healthy, untreated mice of approximately the same age. A comparison among all categories was carried out through a one-way analysis of variance (GraphPad Prism software, version 5.0). There were no statistically significant differences in the SGOT levels in any group of mice treated with anti-*T. cruzi* agents or placebo.

DISCUSSION

Benznidazole and nifurtimox are the only anti-*T. cruzi* drugs available on the market for human use, although they are not fully effective against all disease stages. They also cause undesirable toxic effects that range from mild to severe and may even lead to the interruption of treatment (20, 34).

In our study, NFOH was a successful treatment against *T. cruzi* in the murine model used. In terms of reducing parasite loads to undetectable levels, as measured by direct parasitemia and PCR, NFOH was at least as effective as BZL (Fig. 2, Table 1, and Fig. 4). The level of antibodies is the most reliable parameter to determine the cure of Chagas' disease, since antibodies remain circulating in peripheral blood in response to even minimal or undetectable infection (11). Mice treated with NFOH displayed low serological values 6 months after the completion of treatment. Although these titers were slightly higher than those seen after a month, they all were near the cutoff value (Fig. 3A and B). At both time points, the antibody titers of BZL-treated mice were slightly lower than those of mice that received NFOH, although there were no statistically significant differences between them. In spite of its high initial level of parasitemia, one of the placebo-treated mice was PCR negative at 30 and 180 days posttreatment but displayed high levels of anti-*T. cruzi* antibodies. This result suggests that the mouse was indeed infected and had entered the chronic phase without any further release of parasites to the blood. No amastigote nests were seen on the histological sections analyzed, but mice that received placebo displayed advanced signs of infection (lymphomonocytic infiltrates and nuclear dysplasia in skeletal muscle) that were absent in treated mice (Fig. 5 and 6). Liver sections showed the lowest degree of damage in the NFOH group and the highest in the BZL group. NF- and placebo-treated mice (Fig. 6) displayed intermediate lymphomonocytic infiltrates and necrotic areas in the liver.

In clinical practice, treatment interruption due to the adverse effects of BZL in adult humans is advised when transaminases show high values (34). In contrast, newborns and young children display a higher tolerance to BZL. Although in the

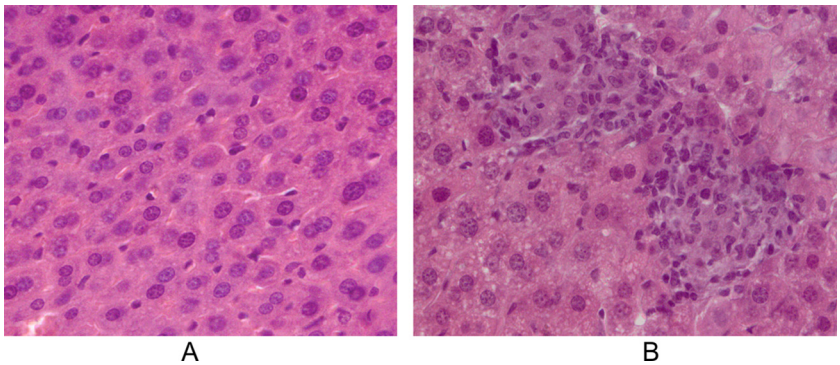

FIG. 6. Liver sections. (A) Healthy liver tissue; (B) focal inflammatory infiltrate of an infected, untreated mouse. Hematoxylin-eosin staining. Magnification, ×400.

present work the hepatic enzymes did not show altered values, evidence of toxicity was given by histological damage in the liver and the rates of mortality among the mice receiving the different treatments.

Nitroheterocyclic compounds, a group that includes NFOH, are metabolically reduced to form a nitroanion, which causes DNA damage and subsequent cell killing (19, 25). In 1988, Henderson and colleagues (17) demonstrated that these compounds, in particular, nitrofurans, under anaerobic conditions inhibited trypanothione reductase, a key detoxifying enzyme of *T. cruzi*. The nitrofuran nifurtimox has been shown to impair the specific cell-mediated immune response of the mammalian host (21, 22) and also displayed toxicity to the peripheral nervous system, caused ovarian and testicular injury, and showed more severe mutagenic effects than benznidazole (6). In the search for better drugs, the rational design of molecules is applied to generate compounds with different substitutions in the nitrofuran and benzimidazole molecules, and these have been tested for their trypanocidal activities (2, 10, 23). "Latentiation" refers to a molecular modification of a drug from its native form to that which, by chemical or enzymatic reactions occurring *in vivo*, will release the active portion of the molecule near its target site (8, 30). Rational design and latentiation led to the design of NFOH, which acts as a prodrug with strong *in vitro* anti-*T. cruzi* activity, being twice as active as the parent compound, NF (8, 15). NFOH is reported to be four times less mutagenic in the Ames test than its parent compound (15), and it has been postulated to have a dual mechanism of action (31). Like a typical nitrofuran, NFOH can inhibit the *T. cruzi* trypanothione reductase, releasing the toxic superoxide anion that ultimately causes DNA damage and cell death. Moreover, NFOH is also able to inhibit 60% of cruzipain activity, whereas nitrofurazone produces only 30% inhibition (31). This new property also suppresses the metabolic pathways that involve the major *T. cruzi* cysteine protease.

The higher water solubility of NFOH, a result of the hydroxymethyl substitution, facilitated its oral administration to mice. Hydroxylation and methylation are the two first detoxifying cycles in hepatocytes, and this might explain the reduced toxicity of NFOH compared to that of NF, measured in terms of the longer survival of the infected mice treated with NFOH.

Future work with this promising new anti-*T. cruzi* drug will involve tests in the chronic phase of the disease, as well as toxicity assays in mice to assess its toxicity potential.

In conclusion, NFOH behaves like BZL in that it has a curative effect on experimental Chagas' disease in the acute phase of a murine model of infection. In this model, NFOH and BZL treatments led to comparable survival and cure levels. Therefore, NFOH emerges as a promising candidate for further tests as an anti-*T. cruzi* agent.

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