A randomized placebo-controlled study of oxidosqualene oxidase inhibitors' efficacy and safety in treating Chagas disease in a murine model

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I have neither given nor received assistance with this work:

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Abstract:

OBJECTIVE: To establish the efficacy and safety of oxidosqualene cyclase inhibitors (OCIs) in treating *Trypanosoma cruzi* infection (Chagas disease) in a mouse model as reflected by extent of parasitemia, survival, and liver enzyme changes through comparison to placebo therapy and standard nifurtimox treatment.

METHODS:

Design: A randomized, experimental, active- and placebo- controlled trial.

Setting: The Animal Research Center at the University of Michigan College of Pharmacy

Subjects: Healthy NHRI mice, as described in the Vertebrate Animals section of this paper.

Interventions: In the efficacy arm of the study, *T. cruzi*-infected mice will be administered an OCI (two types total will be tested), nifurtimox, or vehicle alone, in all cases orally and at a drug concentration and rate of 50mg/kg/d. In the trial's safety arm, mice will receive the same treatments or placebo, except that four additional groups of mice will be administered 25mg/kg/d or 75mg/kg/d of an OCI.

RESULTS:

Measurements: Treatment will continue for 42 days, during which time blood levels of trypomastigotes will be determined weekly starting 7 days post-infection. Survival will be assessed daily. Safety – as reflected by liver alterations – will be ascertained via serum glutamic oxaloacetic transaminase (SGOT) level measurement 30 days post-treatment. Mice showing accepted signs of toxicity will be removed from the study and euthanized as necessary.

Expected results: Groups undergoing treatment with OCIs and nifurtimox should have lower mean blood parasite levels and greater survival than those administered placebo; OCI-treated mice should, furthermore, exhibit lower blood SGOT levels than animals administered nifurtimox.

CONCLUSIONS: The investigation should demonstrate that OCIs provide mice similar protection against increases in blood parasite levels and death to an accepted treatment when each is compared to placebo, while inducing less liver alteration. These findings are important because current therapies, though often effective, frequently pose substantial risks to the patients taking them.

Key Words: *Trypanosoma cruzi*, nifurtimox, lanosterol, epidemiology, acute-phase

Introduction:

Chagas disease exacts a consistent toll on Central and South America, killing roughly 15,000 of an estimated 12 million infected persons yearly (1, 2). The protozoan (*Trypanosoma cruzi*) infection brings minimal acute-phase symptoms, but within 10 to 30 years, 30 to 40 percent of patients enter the chronic phase (3, 4). This stage's common features include heart involvement and organ enlargement; cardiac drugs, defibrillators, and other interventions may provide symptomatic relief to patients who can afford them (5). As Latin American immigrants move globally, they may spread the disease via transfusion or organ donation, while congenital infection also occurs (5, 6).

Currently, only benznidazole and nifurtimox have gained acceptance as standard Chagas disease treatments (6). Used on infants with the acute illness, they affect a cure nearly 100 percent of the time; in children and adults, the value is near 60 percent (7). The agents' use also

comes with adverse effects, often in a majority of patients: benznidazole can cause myalgias, polyneuritis, and agranulocytosis, while nifurtimox can lead to digestive, neurologic, and psychiatric sequelae (7, 8).

Given these efficacy and safety limitations, active seeking of alternatives that eliminate Chagas infection while still in the acute phase is warranted. Ergosterol biosynthesis inhibitors (EBIs) represent some of the newest agents (9). Since trypanosomes cannot take in host cholesterol, their blockade of 24-alkyl sterol formation proves fatal (9). Agents investigated include triazole-derivatives, which block an early demethylation step, and squalene synthase inhibitors (6, 10). These agents' efficacy in murine models has been promising, leading to at least one planned clinical trial (9). A final class, that of oxidosqualene cyclase inhibitors (OCIs), acts on the enzyme that produces lanosterol, the initial precursor of all trypanosomal, mammalian, and fungal steroid structures (11). Buckner and colleagues found significant *in vitro* growth inhibition and high potency among several OCIs tested. Another team subsequently found considerable OCI-generated inhibition of *T. cruzi* cyclase in infected yeast, but murine and other vertebrate-based *in vivo* studies are lacking (12, 13). Such OCIs may be unlikely to affect steroid metabolism in mammals: evidence suggests that humans, at least, compensate for putative inhibition of their own synthesis through enhanced lipoprotein receptor expression (13). Additionally, high-specificity OCI subtypes have been isolated – such agents appear to act on *T. cruzi* at concentrations 100 times less that those needed to affect human fibroblasts, offsetting this potential drawback (3).

It is thus **hypothesized** that administration of an OCI will yield a similar acute-phase Chagas disease cure rate to that brought about by use of the standard treatment nifurtimox in infected mice when each is compared to placebo, and do so with fewer adverse effects.

The hypothesis will be tested by the following specific aims:

1) To assess the efficacy of OCIs by comparing blood parasite levels in and survival of Chagas disease-infected mice treated with one of two oral experimental OCIs or nifurtimox to the same parameters in mice given a placebo treatment using an approved statistical approach.

2) To determine and compare OCI safety, as reflected by liver enzyme level changes, in a Chagas disease-infected murine model treated with either of two oral OCIs, with placebo, or with the standard treatment nifurtimox.

Study Design:

This investigation will take the form of a randomized, experimental, placebo-control study. Approval from the University Committee on Use and Care of Animals (UCUCA) will be obtained before commencing the study and the rules of the committee will be adhered to throughout. Changes to protocol will be submitted to the group for approval before implementation.

Methods:

From a pool of 120 normal, healthy outbred female NHRI mice (weight = 20-25g), individuals will be randomly selected for placement into one of four groups (two control and two experimental, described below) of 30 mice each (13). Each mouse will be infected with 10^5

Trypanosoma cruzi blood trypomastigotes of the Tulahuen strain via intraperitoneal injection (13). Oral treatment with the respective agents will be performed by members of the research team 24 hours post-infection and continue daily for 30 days (13). This route was chosen based on demonstrated inhibition of OSC by oral agents in murine cholesterol studies (14, 15). For assessment of **parasitemia** (a primary efficacy endpoint)**,** 10ul of fresh blood will be examined under a light microscope at 400X magnification every seven days from days 7 to 42 after infection. Enumeration of the parasites in 100 fields will be performed and concentrations will be calculated in trypomastigotes per milliliter (16). Mixed venous-arterial blood for the parasitemia assessment will be obtained from mice's tails via cutting of 1-mm segments, following common protocol (16, 17). **Percent survival** (the second primary efficacy endpoint) will be assessed daily by observation, with mouse status being recorded as dead or alive.

For assessment of toxicity, 180 mice of the strain described above will be randomly divided into six separate groups of 30 mice each and administered either the control or experimental treatments. Activity of the enzyme serum glutamic oxaloacetic transaminase (SGOT) will be assessed at the 30-day post-treatment time point. Increased activity of the enzyme reflects liver alterations in response to long-term anti-*T. cruzi* treatment, which should manifest themselves by the one-month time point (16). Kits will be used for this measurement and are based on appearance of pyruvate, which reacts with 2,4-dinitrophenylhdracine to form a detectable colored compound: the test itself will entail the drawing of 50ul of serum (also obtained by tail cutting); addition of this to a SGOT substrate and incubation at 37°C without and then with 2,4-dinitrophenylhdracine; stopping of the reaction using basic solution and incubation; and subsequent measurement of spectrophotometric absorbance at 505nm (16). Use

of standards to generate a calibration curve will permit determination of pyruvate concentrations in mM via extrapolation (16).

Control groups:

Negative control groups (n=30 for efficacy and toxicity) in both arms will receive orally the vehicle that will be used for delivering the drug treatments, which will consist of 1% methylcellulose and 0.5% Tween 80 (13). Positive control groups of equivalent size will be administered 50mg/kg/d of the reference drug nifurtimox over the same time course as experimental treatments (daily for 30 days starting 24 h post-infection). Monitoring of parasitemia on a weekly basis and daily inspection for survival will occur as described above for mice in the efficacy trial.

Experimental groups:

Mice in each experimental group will receive one of two **oxidosqualene cyclase inhibitor (OCI)-based treatments** via the oral route. Specifically, N-(4E,8E)-5,9, 13-trimethyl-4,8, 12-tetradecatrien-1-ylpyridinium and another unnamed phenyl-derived compound which we have designated A232 will be administered, based on their relatively specific inhibition of *T. cruzi* oxidosqualene cyclase in *in vitro* and *Saccharomyces cerivisiae* studies (3, 12). For efficacy assessment, the agents will be mixed into the vehicle of 1% methylcellulose and 0.5% Tween 80 and administered at a concentration of 50mg/kg/d, the same level as has been used previously for the related squalene-synthase inhibitors, for 30 consecutive days starting 24 h after infection (13). Parasitemia and mortality will be assessed at the same intervals and for the same length of time as for the control groups. A similar drug protocol will hold for the safety analysis, except that each of the two OCIs will be administered according to two different regimens: 25mg/kg/d

and 75mg/kg/d (yielding a total of four experimental groups). These drug doses were chosen to bracket the putative therapeutic dose.

Statistical plan:

Data will be collected over 42 days. Based on microscopic counts, average blood parasite concentration and percent survival (ratio data) across all surviving animals in each group will be calculated and plotted against days post-infection (p.i.). Parasitemia and percent survival are thus the **primary endpoints** of the efficacy analysis**.** We will employ a two-tailed, independent-sample Student's t-test ($p \leq 0.05$) to compare mean experimental-group blood parasite concentration and survival to those of placebo at 21 and 42 days p.i. Comparison to placebo rather will be undertaken due to the excessive number of mice needed to achieve sufficient power to detect the small difference expected if comparison were to active control. The sample size of 120 mice for the efficacy arm will permit detection of a 50 percent difference in parasitemia and survival where $\beta = 0.8$ and $\alpha = 0.05$.

Activity of SGOT, as manifested by pyruvate levels, will form the **primary endpoint** of the safety analysis. The ratio data of mean millimolar pyruvate concentration among each group's surviving mice will be calculated at 30 days only; means will be compared to each other and to levels in the negative control mice via ANOVA analysis ($p \leq 0.05$). Analysis of data from both efficacy and safety analyses will be conducted using IBM's SPSS program version 19. Results will be displayed in a table as well as in graphical form with error bars. The 180-mouse sample size was selected to permit discernment of a 10 percent difference in pyruvate concentration where $\beta = 0.8$ and $\alpha = 0.05$.

Issues related to use of vertebrate animals:

1) *Description of procedures* In the study we will use a total of 300 outbred female **NMRI mice** (Charles River, Wilmington, MA) to advance aims #1 and #2, regarding the safety and efficacy of the experimental treatments**.** Mice of this sex and variety are readily available, lend themselves well to biomedical purposes, and have been used effectively in previous murine *in vivo* studies of agents targeting the cholesterol biosynthesis pathway of *Trypanosoma cruzi* (2, 13).

Withdrawing of blood samples from the tail: As tail blood sampling requires that mice be placed under anesthesia, each surviving mouse will undergo isoflurane administration using a cotton gauze and zipper-sealed plastic bag following methods documented in the literature (17). Blood sampling will entail the removal of 1 to 2 mm segments of tail using a sharp scalpel followed by capillary tube use for droplet collection, in accordance with accepted protocol (17). Upon recovery, mice will be returned to their cages and allowed to roam, eat and drink at will. Oral administration of all drug treatments subjects the animals to minimal discomfort and will occur while the animals are awake.

2) *Justification of* use: The use of animals is required for the testing of our safety and efficacy hypotheses because of the absence of *in vivo* studies on OCIs. A murine model, in particular, comprises our model of choice due to the high degree of similarity between the human and mouse genome, small size and thus eased housing and maintenance of the animals, and their ready availability. We have established the number of animals needed for reasons outlined in the Statistical Plan, above, and our desire to use as few animals as possible while still providing flexibility should some individuals require removal on grounds unrelated to study interventions.

3) *Caring for the animals*: Feeding, housing, and other husbandry of the animals will be undertaken by members of the Animal Research Center at the University of Michigan. Supervisors will have undergone training and carry certification from the American Association for Laboratory Animal Science. Should animals require care, veterinary residents will render it.

4) **To keep discomfort, injury, pain and stress levels of the animals at the lowest possible level**, mice will undergo anesthesia before every tail-cutting/blood-sampling procedure. The one-week interval between samplings provides sufficient time for mouse recovery from tail cutting and minimizes the number of times the animals are exposed to anesthetic. Any drug treatment and/or blood sampling will be ceased in mice displaying signs of drug toxicity or excessive stress such as erected hair, dehydration and inactivity (16). **Euthanasia** via barbiturate overdose (IV administration of sodium pentobarbital at >/=100mg/kg) and according to American Veterinary Medical Association stipulations will be utilized should one or multiple criteria for mouse removal from the study (such as weight loss, moribund state, or infection) are found based on the opinion of a veterinary consultant.

Conclusions, Limitations, and Future Directions

The mortality and morbidity caused by Chagas disease in endemic regions of South and Central America is high, with millions of individuals in the acute or chronic stages of the disease at any time. Current therapies are moderately effective, but often cause significant toxicity, enhancing the need for better treatments. By inhibiting trypanosomal synthesis of key cholesterol derivatives, oxidosqualene cyclase inhibitors should provide mortality- and blood parasite level-decreasing ability on par with that of standard treatment relative to placebo, while causing significantly less hepatic impairment.

Limitations to the current study include the assumption of an appropriate dose that, while chosen based on studies of similar agents, may not prove adequate in treating the infection. Examination of liver enzyme changes may fail to fully capture manifestations of toxicity such as impairment of murine cholesterol synthesis and its effects.

Additional studies of longer duration may focus on the utility of OCIs in preventing progression of the disease to the chronic stage. A broader range of OCIs could also be employed in treatment, and toxicity could be monitored through the analysis of a broader range of parameters simultaneously. Should their safety and efficacy prove high, use of these agents may be extended into early-phase clinical trials.

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