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Evaluation of benznidazole treatment combined with nifurtimox, posaconazole or AmBisome® in mice infected with *Trypanosoma cruzi* strains

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ABSTRACT

The present work aimed to investigate the curative effect of benznidazole (BZL) in combination with other patented drugs [nifurtimox (NFX), posaconazole (POS) or AmBisome® (AMB)] in mice acutely or chronically infected with either a BZL-susceptible (Tulahuen) or a BZL-partially-resistant (Y) strain of *Trypanosoma cruzi*. To appreciate the eventual advantage of such combinations, infected mice were treated for short durations (non-curative) of each individual treatment. Cure rates were determined by investigating blood parasites (microscopic examination) and parasite DNA (quantitative PCR) after submitting treated mice to immune suppression with cyclophosphamide. The results mainly suggest that shorter durations of treatment combining BZL and POS or NFX might cure mice acutely or chronically infected with the Tulahuen strain, whereas the combination of BZL with AMB does not have such an effect. Moreover, the association BZL + POS does not improve the curative effect of POS (all used for shorter durations) in infection with the Y strain. Shortening the duration of treatment whilst keeping a complete curative effect deserves interest in limiting adverse reactions due to dose-cumulative toxic effects of long treatment. Genotyping of the *T. cruzi* strain(s) infecting patients might also allow a better adaptation of individual therapeutic schedules, improving both the efficiency and safety of trypanocidal treatment. This preliminary experimental study should encourage further investigations to find the best combination of adequate drug concentrations and timing of treatment.

1. Introduction

Chagas disease, caused by the kinetoplastid flagellate *Trypanosoma cruzi*, is one of the major causes of cardiac failure in Latin America [1]. This neglected tropical disease has become a global public health problem due to Latin American migrations to non-endemic countries, particularly the USA, Europe, Japan and Australia [2]. Following an acute phase, generally asymptomatic although sometimes fatal in children, the infection evolves to an asymptomatic and silent chronic phase. Amongst infected individuals, 30–40% develop symptomatic cardiac (the most frequently encountered) and/or digestive clinical forms of chronic Chagas disease (megacolon and/or megaoesophagus), responsible for significant morbidity and mortality, decades after primary infection [1].

The currently used trypanocidal drugs, namely the nitroimidazole benznidazole (BZL) and the nitrofuran nifurtimox (NFX), are effective in acute infection (particularly congenital Chagas disease), re-activated infection (in the case of immune suppression) and the early chronic phase of infection [3,4]. Their efficacy diminishes in the late chronic phase, although BZL appears to prevent progression of cardiac lesions when administered to chronically infected patients [5]. Natural resistance of some *T. cruzi* strains to nitroderivatives might lower the cure rates in treated chagasic patients [6,7]. Such drugs have to be taken orally for 1–3 months and cause adverse reactions in up to 40% of treated adult patients. Some side effects (such as exfoliative dermatitis or peripheral polyneuritis) are sufficiently severe to stop treatment [8], and late side effects are likely due to the cumulative toxic effects of these drugs given over a long period [4,8,9]. Thus, chemotherapy of Chagas disease remains an unsolved problem.

A number of different compounds have been assessed as alternative treatments for *T. cruzi* infection [4,10]. As ergosterol is the predominant sterol of the parasite membrane [11], several inhibitors of ergosterol synthesis, initially developed for the treatment of invasive fungal infections, have appeared as alternatives. One of these inhibitors, namely posaconazole (POS), a triazole derivative that potentially inhibits the course of *T. cruzi* infection in mice, is presently undergoing clinical trials [4,12]. Another compound, AmBisome® (AMB) (a liposomal formulation of the macrolide polyene amphotericin B), displays a high affinity for

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ergosterol and is also a current and potent treatment of invasive fungal infections as well as visceral leishmaniasis. We, along with others, have shown the efficiency of AMB in preventing mortality and drastically reducing parasitaemia in mice infected with *T. cruzi* [13,14], although a complete cure could not be achieved [13].

Another alternative for the treatment of *T. cruzi* infection might be the combination of drugs having some capacity to control parasite multiplication [10]. This approach might improve the efficacy of treatment (by generating additive or synergistic effects on different pharmacological targets) and decrease the likelihood of drug resistance and toxic effects (by reducing the drug dosages or treatment durations used). Several associations of two drugs inducing limited improvements in mice [15–20] or in patients [21] have been tested. However, as far as we know, no experimental studies or clinical trials combining the already patented drugs available on the market (BZL, NFX, POS and AMB) have been reported.

Here we evaluate the effect of BZL, in combination with these other drugs, in mice acutely or chronically infected whether with a BZL-susceptible (Tulahuen) or a BZL-partially-resistant (Y) strain of *T. cruzi* [6,7]. To appreciate the eventual advantage of such combinations, drugs were given for shorter durations than usually used. Cure rates were determined after submitting treated mice to immune suppression with cyclophosphamide (CP) by verifying the absence of reactivation of any cryptic infection by microscopic examination of blood as well as quantitative PCR (qPCR).

2. Materials and methods

2.1. Drugs

BZL (Lafepe, Recife, Brazil) and NFX (Lampit®; Bayer S.A., San Salvador, El Salvador) were obtained from the World Health Organization (Geneva, Switzerland). POS (Noxafil®) and AMB were purchased from Schering-Plough (Brussels, Belgium) and Gilead (Paris, France), respectively. BZL and NFX tablets were crushed and suspended in distilled water, whilst POS was directly suspended in distilled water. Combination of BZL with NFX or POS consisted of a mixed suspension of both drugs.

2.2. Mice, infection and treatment schedules

BALB/cJ female mice (six to eight mice per studied subgroup) were obtained from Janvier (Le Genest-St-Isle, France) and were maintained under a 12-h dark/light cycle (lights on at 06:30 h) at a temperature of 22 ± 3 °C with free access to standard chow and drinking water in accredited animal facilities.

Mice were infected when they were 7 weeks old by intraperitoneal (i.p.) injection of 1000 blood trypomastigotes of Tulahuen (TcVI genotype) or Y (TcII genotype) strains of *T. cruzi*, known to be susceptible and partially resistant to BZL, respectively [6,7]. Mortality was recorded daily and parasitaemia was determined regularly by microscopic examination of tail vein blood of infected animals, with a detection limit of 10 000 parasites/mL [22].

Infected animals were divided into nine groups: non-treated (NT); mice treated with BZL at a curative standard schedule for the Tulahuen strain (BZLo) or for a reduced suboptimal period (BZLso); mice treated with NFX, POS or AMB for suboptimal durations (NFXso, POSso and AMBso, respectively); and mice treated with BZLso + NFXso, BZLso + POSso or BZLso + AMBso. Table 1 indicates the routes of administration, doses and tested durations of applied treatments as well as the related references of standard (optimum) treatments. Animals treated with BZL, NFX or POS received 0.2 mL of single drug suspension or drug combination by oral gavage, whereas AMB was injected by the i.p. route. Treatments in the acute phase started at the onset of parasitaemia [5 days and 10 days post inoculation (dpi) for Y and Tulahuen strains, respectively] and those of chronically infected mice started at 60 dpi.

2.3. Cyclophosphamide-induced immune suppression and assessment of cure

The general design of the experiments is shown in Fig. 1. Briefly, 1 week after the end of treatment, parasitaemia was checked and mice negative by fresh blood microscopic examination received 4 i.p. injections of 200 mg/kg CP (Endoxan; Baxter, Lessines, Belgium) on alternate days as previously described [13,23]. The efficacy of such an immunosuppression procedure to assess cryptic infection was verified by the high parasitaemias (microscopic examination) and mortality of 100% of chronically untreated (NT) mice having received CP. Within 2 weeks after the last CP injection in treated mice, the presence of blood trypomastigotes was checked by microscopy. Mice negative for such microscopic determination were submitted to retro-orbital puncture (under gaseous anaesthesia) to collect blood in citrated microtubes to investigate the presence of parasite DNA by qPCR.

Detection of blood parasites or *T. cruzi* DNA in a treated mouse, before or after CP-induced immune suppression, indicated treatment failure. By contrast, a qPCR-negative result in the blood of a treated and immunosuppressed mouse confirmed the absence of parasites, indicating complete cure of the animal. Higher cure rates in the groups submitted to combined treatments than in animal groups having received only one drug were considered indicative of a cumulative effect of drug combinations compared with drugs used alone.

2.4. DNA extraction and quantitative PCR

DNA extraction was performed as described previously [13] on 200 μL blood samples using GeneMole apparatus and DNA Blood

Table 1
Routes of administration, doses and durations of tested treatments.

Drug	Phase of infection	Route, dose (mg/kg/day) (reference)	Duration (days)
BZLo	Acute	p.o., 100 [25][a]	20[b]
	Chronic	p.o., 100 [23][a]	10[b]
BZLso	Acute	p.o., 100 [25][a]	10[b]
	Chronic	p.o., 100 [23][a]	5[b]
NFXso	Acute	p.o., 100 [29]	10[b]
	Chronic	p.o., 100 [30]	5[b]
POSso	Acute	p.o., 20 [12]	10[b]
	Chronic	p.o., 20 [25]	5[b]
AMBso	Acute	i.p., 25 [13]	5[c]
	Chronic	i.p., 25 [13]	5[b]

BZL, benznidazole; NFX, nifurtimox; POS, posaconazole; AMB, AmBisome®; o, optimal treatment duration; so, suboptimal treatment duration; p.o., per os; i.p., intraperitoneal.
[a] Also based on previous extensive experiments we previously performed in our laboratory (unpublished results).
[b] Treatment given on consecutive days.
[c] Treatment given on alternate days.