Parasitology

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Combination therapy using nitro compounds improves the efficacy of experimental Chagas disease treatment

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Research Article

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Abstract

Drug combinations have been evaluated for Chagas disease in an attempt to improve efficacy and safety. In this line, the objective of this work is to assess the effects of treatment with nitro drugs combinations using benznidazole (BZ) or nifurtimox (NFX) plus the sulfone metabolite of fexinidazole (fex-SFN) *in vitro* and *in vivo* on *Trypanosoma cruzi* infection. The *in vitro* interaction of fex-SFN and BZ or NFX against infected H9c2 cells by the Y strain was classified as an additive ($0.5 \geqslant \Sigma FIC < 4$), suggesting the possibility of a dose reduction in the *in vivo* *T. cruzi* infection. Next, the effect of combining suboptimal doses was assessed in an acute model of murine *T. cruzi* infection. Drug combinations led to a faster suppression of parasitemia than monotherapies. Also, the associations led to higher cure levels than those in the reference treatment BZ 100 mg day^{-1} (57.1%) (i.e. 83.3% with BZ/fex-SFN and 75% with NFX/fex-SFN). Importantly, toxic effects resulting from the associations were not observed, according to weight gain and hepatic enzyme levels in the serum of experimental animals. Taken together, this study is a starting point to explore the potential effects of nitro drugs combinations in preclinical models of kinetoplastid-related infections.

Introduction

Chagas disease is caused by the protozoan *Trypanosoma cruzi* (Chagas, 1909) and affects about 7 million people worldwide, especially in the 21 endemic countries of Latin America (WHO, 2020). The infection is classified as a neglected tropical disease and related to poor populations in tropical and subtropical regions, although it has been spread to non-endemic areas in Europe, the USA and Japan (Lidani *et al.*, 2019). Benznidazole (BZ) and nifurtimox (NFX) are the nitro drugs of choice for the treatment of *T. cruzi* infection. These drugs are effective in inducing cure in the early stages of infection, but the benefit of their administration in the chronic phase is limited due to variable efficacy (Ribeiro *et al.*, 2020). In addition, treatment is long and may lead to several adverse reactions that compromise its continuation (Pérez-Molina and Molina, 2018).

Looking for new alternatives of Chagas disease treatment, a large number of molecules have been evaluated in preclinical studies and few promising compounds have been tested in clinical trials (Villalta and Rachakonda, 2019), including the azolic inhibitors of sterol C14α demethylase posaconazole and fosravuconazole (Molina *et al.*, 2014; Torrico *et al.*, 2018). Although these clinical studies represented an advance in the field of chemotherapy for Chagas disease, they led to disappointing results, with high levels of therapeutic failure detected (Molina *et al.*, 2014; Morillo *et al.*, 2015; Torrico *et al.*, 2018). While limitations concerning the appropriate dose and duration of treatments with azoles required to control human *T. cruzi* infections are being discussed (Martínez-Peinado *et al.*, 2020), the clinical development pipeline for Chagas disease is actually based on nitro compounds, either in proof of concept trials, such as fexinidazole (FEX12-NCT03587766), or evaluating new regimens of compounds already in use (MultiBenz – NCT03191162; BENDITA – NCT03378661; CHICAMOCHA 3 – NCT02369978; NCT03981523) (Martínez-Peinado *et al.*, 2020).

Nitro compounds, such as the nitrofuran NFX and the nitroimidazoles BZ and fexinidazole, are a group of bioactive compounds with well-established indications to treat a wide variety of conditions, including those caused by parasites (Patterson and Wyllie, 2014). The basis for their biological activity is the biotransformation of the nitro group, releasing intermediates in the redox process, which bind to macromolecules causing damage (Patterson and Fairlamb, 2019).

Particularly, fexinidazole is very active against trypanosomatides (Winkelmann and Raether, 1978; Jennings and Urquhart, 1983; Raether and Seidenath, 1983; Kaiser *et al.*, 2011; Bahia *et al.*, 2012; Wyllie *et al.*, 2012; Tarral *et al.*, 2014), and the drug was recently approved as the first oral treatment for African trypanosomiasis (Deeks, 2019). After

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promising preclinical data on *T. cruzi* infection, fexinidazole has been evaluated in clinical trials against chronic Chagas disease (DNDi, 2020). The first clinical trial was interrupted due to safety and tolerability issues (NCT02498782). The second clinical trial, using lower fexinidazole doses, has now been completed (FEX12 – NCT03587766), but the results are not available (DNDi, 2020). Fexinidazole is orally available and is rapidly converted by oxidative metabolism to two metabolites, the sulfoxide and the sulfone (Torreele *et al.*, 2010). Bahia *et al.* demonstrated that the oral administration of fexinidazole metabolites was well tolerated and effective in treating acute murine *T. cruzi* infection (Bahia *et al.*, 2014). The authors identified that the primary effective species is probably the sulfone metabolite of fexinidazole (fex-SFN) and that high concentrations need to be maintained to ensure efficacy (Bahia *et al.*, 2014). However, the effects of lower doses of this metabolite when combined with other drugs have not been evaluated yet.

Drug combinations have been evaluated for experimental (Araujo *et al.*, 2000; Benaim *et al.*, 2006; Cencig *et al.*, 2012; de Diniz *et al.*, 2013, 2018; Grosso *et al.*, 2013; Strauss *et al.*, 2013; Assíria Fontes Martins *et al.*, 2015; Providello *et al.*, 2018; Guedes-da-Silva *et al.*, 2019; Mazzeti *et al.*, 2019; Rocha Simões-Silva *et al.*, 2019; Machado *et al.*, 2020; Ribeiro *et al.*, 2020) and in the clinical context for Chagas disease (BENDITA – NCT03378661, STOP CHAGAS – NCT01377480). Interestingly, the association between nitro compounds has not been extensively evaluated. Thus, the objective of this work is to assess the effects of treatment with BZ, NFX, fex-SFN, and their combinations *in vitro* and *in vivo* on experimental *T. cruzi* infection.

Materials and methods

Parasite

In the study, the *T. cruzi* Y strain classified as DTU II (Zingales *et al.*, 2009) and previously characterized as partially resistant to BZ (Filardi and Brener, 1987) was used.

Study drugs

BZ, which is also known as N-benzyl-2-(2-nitroimidazol-1-yl) acetamide, was purchased from *Laboratório Farmacêutico de Pernambuco* (LAFEPE, Recife, Brazil). Fex-SFN, which is also known as 1-methyl-2-(4-methylsulfonyl phenoxymethyl)-5-nitroimidazole (Axyntis/Centipharm, France), was provided by the Drugs for Neglected Diseases *initiative* (DND*i*). NFX, which is also known as (E)-N-(3-methyl-1,1-dioxo-1,4-thiazinan-4-yl)-1-(5-nitrofuran-2-yl) methanimine, was donated by the DND*i*.

For *in vitro* assays, stock solutions of BZ, fex-SFN and NFX were prepared in dimethyl sulfoxide (DMSO) and stored at −20°C. Stock solutions were further diluted to appropriate working concentrations using a culture medium. Importantly, the final DMSO concentration never exceeded 0.5% (v/v) in order to avoid toxicity to host cells. For *in vivo* assays, BZ and NFX were suspended in 0.5% methylcellulose solution in distilled water and fex-SFN was formulated in an aqueous suspension containing 0.5% methylcellulose and 5.0% polysorbate.

In vitro *assays*

Toxicity of combinations of nitro compounds to host cells

In vitro assays were performed using the H9c2 (American Type Culture Collection, ATCC: CRL 1446) cardiomyoblast lineage. Cells were maintained in 25 cm^2 bottles in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% l-glutamine 2 μM, 100 IU mL^{-1} penicillin and 0.1 mg mL^{-1} streptomycin at 37°C in an atmosphere of 5% CO_2. To exclude toxic effects of drug combinations on mammalian cells, cytotoxicity was measured by the Resazurin colorimetric assay for cell viability and proliferation inhibition according to the previously described method (Diniz *et al.*, 2018). Briefly, 1×10^3 H9c2 cells were plated per well in 96-well plates and incubated at 37°C, 5% CO_2 for 24 h. Thus, cells were incubated for 72 h with increasing concentrations of each drug in twofold dilutions, covering a range of 1.56–200 μM for BZ, 0.78–100 μM for NFX and 1.56–200 μM for fex-SFN. The top concentration of combined treatment was 100 μM plus 100 μM for BZ/fex-SFN and 50 μM plus 100 μM for NFX/fex-SFN, followed by eight 1:2 dilutions. The plates were subjected to microplate spectrophotometer reading (Biochrom Anthos 2010 Microplate Reader, Cambridge, United Kingdom) at 570 and 600 nm wavelengths. The percentage of cell viability induced by the treatments was calculated considering the percentage of reduction of incubated cells in the absence of drug. All tests were performed at least twice in triplicate, and the reduction of cell viability by more than 30% was considered cytotoxic, as recommended by the International Organization for Standardization (ISO, 2009).

Determination of nature of interaction among nitro compounds

The evaluation of *in vitro* anti-amastigote activity was performed using H9c2 cells infected with the Y strain, according to Diniz *et al.* (2018). For this, 1×10^4 cells were seeded on coverslips in 24-well plates; after 24 h, cells were infected with *T. cruzi* Y strain trypomastigotes (20:1 ratio of parasites to host cells). Non-adherent parasites were removed by washing with DMEM after 24 h of interaction, and the cultures were exposed to compounds alone or in combination at concentrations ranging from 0.15 to 20 μM for BZ, 0.08 to 10 μM for NFX and 0.15 to 20 μM for fex-SFN. After 72 h of incubation, the cultures were fixed with methanol, stained with Giemsa, and examined microscopically to determine the percentage of cells infected in treated and untreated controls. IC_{50} and IC_{90} values were calculated using Calcusyn software (Biosoft, UK). All tests were performed at least twice in duplicate, and the results were given as mean ± standard deviation.

In vivo *assays*

Mice, infection and treatment

Female Swiss mice (18–22 g) from the animal facility at UFOP were maintained in a temperature-controlled room with access to water and food *ad libitum* under 12 h day/night cycles and temperature 22 ± 2°C. Animals were inoculated intraperitoneally with 5×10^3 blood trypomastigotes of *T. cruzi* Y strain and randomly divided into groups of 6–10 animals. As control groups, infected and untreated and uninfected and untreated animals were used. Each treated group received the compounds daily at different doses alone or in combination by oral gavage for 20 days: BZ 100 mg kg^{-1} (reference treatment), BZ 50 mg kg^{-1}, fex-SFN 50 mg kg^{-1}, NFX 25 mg kg^{-1}, and combination therapy consisting of BZ 50 mg kg^{-1} + fex-SFN 50 mg kg^{-1} or NFX 25 mg kg^{-1} + fex-SFN 50 mg kg^{-1}. All treatments began on the fourth day after infection at the onset of parasitemia. Mortality was checked daily until 30 days after treatment.

Determination of treatment efficacy

Treatment efficacy was determined following the methodology of Caldas *et al.* (2008) based on parasitemia detection by fresh blood examination (Brener, 1962) before and after cyclophosphamide (Baxter Oncology, Germany) immunosuppression and blood qPCR. Animals with negative results in the fresh blood examination up to 30 days after treatment were immunosuppressed with cyclophosphamide at the dose of 50 mg kg^{-1} in three cycles

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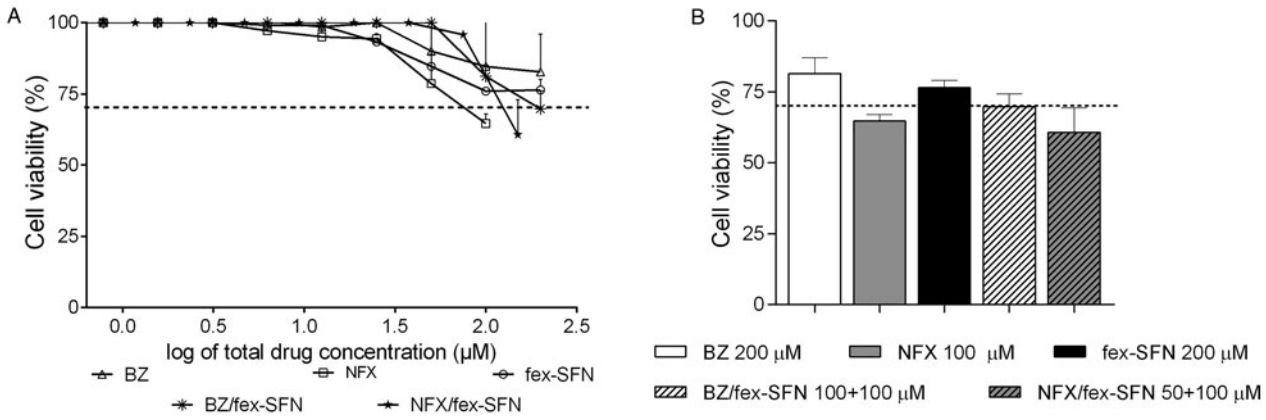

Fig. 1. H9c2 cell viability after 72 h of treatment with benznidazole (BZ), nifurtimox (NFX), sulfone metabolite of fexinidazole (fex-SFN) and their combinations. (A) Effects of nitro drugs alone or in combination on the viability of H9c2 cells upon 72 h incubation at the total top concentrations of BZ (200 μM), NFX (100 μM), fex-SFN (200 μM) and their combinations, including BZ/fex-SFN (100 + 100 μM) and NFX/fex-SFN (50 + 100 μM), and seven twofold serial dilutions. (B) Bar graphic showing cell viability at the top concentrations of nitro drugs and their combinations in the H9c2 cell line. The results are the average of two independent experiments performed in triplicate.

of four consecutive with an interval of 3 days between each cycle. Parasitemia was checked daily during and up to 10 days after immunosuppression cycles.

For qPCR, blood samples were collected 30 and 180 days after treatment from mice with negative fresh blood examinations. Also, heart samples were collected during euthanasia 180 days after treatment to quantify parasite DNA in tissue. The genomic DNA of samples was isolated and purified using the Wizard Genomic DNA Purification Kit (Promega Corp., Madison, WI, USA), according to the manufacturer's instructions. The presence of *T. cruzi* in samples was evaluated by amplifying a 195-bp sequence, repeated in tandem in genomic DNA, using TCZ-F (5-GCTCTTGCCCACAMGGGTGC-3, where M indicates A or C) and TCZ-R (5-CCAAGCAGCGGATAGTTCAGG-3) primers as described by Cummings and Tarleton (2003). The murine TNF-α gene sequence was amplified separately using the primers TNF-5241 (5-TCCCTCTCATCAGTTCTATGGCCCA-3) and TNF-5411 (5-CAGCAAGCATCTATGCACTTAGACCCC-3) (Cummings and Tarleton, 2003). Reactions consisted of 2 μL of template DNA at 25 μg mL^{-1}, specific primers at a final concentration of 10 μM and Sybr-Green PCR Master Mix in a total volume of 10 μL. Standard curves for DNA parasite quantification in the cardiac tissue were produced from 10-fold DNA dilution of epimastigotes of *T. cruzi* Y strain in DNA from the heart tissue of non-infected mice, ranging from 1×10^6 to 1 parasite equivalent/25 μg of tissue DNA. The DNA amplifications were carried out in the 7500 Fast Real-Time PCR System (Applied Biosystems, Life Technologies, California, USA). After the initial denaturation step of 10 min at 95°C, amplification was carried out for 40 cycles (94°C for 15 s). Fluorescence data collection was performed at 62°C for 1 min at the end of each cycle. Amplification was immediately followed by a melting programme with initial denaturation for 15 s at 95°C, cooling to 60°C for 1 min, and then stepwise temperature increases from 60 to 95°C at 0.3°C s^{-1}. All samples were analysed in duplicate, and negative samples and reagent controls were processed in parallel in each assay. The efficiencies of amplification were determined automatically by 7500 Fast Real-Time PCR software. Animals showing negative results in all tests were considered cured.

Determination of treatment toxicity

Weight determination was performed every 7 days up to 30 days after treatment. In addition, treatment toxicity was evaluated by hepatic enzyme dosages in mouse serum collected on the last day of treatment. For infected and untreated mice, the samples were obtained at day 15 post-infection. In this group, subsequent sampling could not be performed as *T. cruzi* Y strain infection induced 100% of mortality until day 18 of infection. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined by colorimetric assay using the commercial Bioclin® kit, according to the manufacturer's instructions, in an autoanalyser (Wiener Lab model CM200 – kinetic analysis).

Statistical analysis

Statistical analysis of the data was performed using GraphPad Prism 5.01 software (GraphPad Software Inc., San Diego, CA, USA). The results were expressed as mean ± standard deviation. Parametric data were analysed with Student's *t*-test, and non-parametric data were analysed with the Mann–Whitney test. Statistical significance was established with 95% confidence intervals and $P < 0.05$.

The nature of *in vitro* drug interactions was determined by the fractional inhibitory concentration (FIC) index. FICs at IC_{50} and the sum of FICs (ΣFICs) were calculated: FIC of drug A = IC_{50} of drug A in combination/IC_{50} of drug A alone. The same equation was applied to the partner drug (drug B), and ΣFICs = FIC drug A + FIC drug B was calculated. The ΣFIC_{50} was used to classify the interaction as synergistic ($\Sigma FIC \leqslant 0.5$), additive or no interaction ($0.5 \geqslant \Sigma FIC < 4$), or antagonistic ($\Sigma FIC > 4$) (Odds, 2003).

Results

Initially, the cytotoxicities of BZ, NFX and fex-SFN were evaluated in our mammalian host cells model, either alone or in combination. Figure 1 shows the cell viability after 72 h of drug incubation. BZ, fex-SFN and their combinations did not interfere with the viability of H9c2 cells at all concentrations tested (Fig. 1A). Only incubation with 100 μM NFX and the highest concentrations of the NFX/fex-SFN combination (50 + 100 μM) induced more than a 30% reduction in cell viability and were considered toxic (Fig. 1B) (ISO, 2009).

The concentrations considered non-toxic were used to investigate the nature of the *in vitro* interaction between fex-SFN and BZ or NFX on H9c2 cells infected with the Y strain and the results were analysed at the IC_{50} level. As expected, the dose-response

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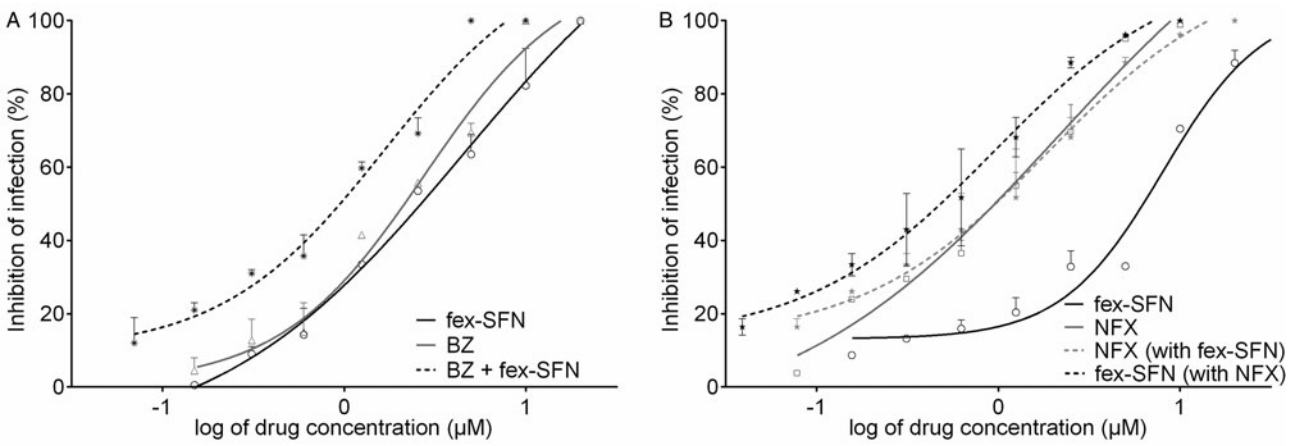

C

Monotherapy	BZ	fex-SFN	NFX
IC_{50} (μM)	1.53 ± 0.52	3.49 ± 1.32	0.65 ± 0.12
IC_{90} (μM)	5.69 ± 0.79	41.68 ± 32.14	3.49 ± 0.06
Combination	**BZ/fex-SNF**		**NFX/fex-SNF**
ΣFIC_{50}	0.675 ± 0.15		0.695 ± 0.20
ΣFIC_{90}	0.758 ± 0.06		1.102 ± 0.65

Fig. 2. Anti-*Trypanosoma cruzi* activity. *In vitro* dose-response curves of H9c2 cells infected with *Trypanosoma cruzi* Y strain treated with nitro compounds in monotherapy or in combination for 72 h. (A) Treatment with fex-SFN, BZ and BZ/fex-SFN. (B) Treatment with fex-SFN, NFX and NFX/fex-SFN. Each point of the dose-response curves corresponds to the mean of two independent experiments. (C) IC_{50} and ΣFIC_{50} of nitrocompounds alone and in combination.

curves showed potent and concentration-dependent effects of the nitro drugs singly against intracellular parasites (Fig. 2). The IC_{50} value for BZ and NFX was 1.53 ± 0.52 and 0.65 ± 0.12 μM, respectively; similarly, the dose-dependent effect of fex-SFN revealed an IC_{50} value of 3.49 ± 1.32 μM (Fig. 2C). When used in combination with BZ or NFX, a leftward shift of the combined therapy curve for fex-SFN was identified, suggesting a positive effect resulting from the drug combinations. This effect was confirmed by the analysis of the ΣFICs that revealed $\Sigma FIC_{50} = 0.675 \pm 0.15$ for the combination of BZ and fex-SNF and 0.695 ± 0.20 for the combination of NFX and fex-SFN (Fig. 2C), indicating an additive effect.

Considering the results of the *in vitro* experiments, we evaluated the effect of the same drug combinations in an acute model of murine *T. cruzi* infection. In this case, suboptimal doses of each drug (i.e. those that did not induce cure in the experimental murine model infection) (Mazzeti *et al.*, 2018) were used in combination (BZ 50 mg kg^{-1}, fex-SFN 50 mg kg^{-1} and NFX 25 mg kg^{-1}). Figure 3 shows the parasitemia curves of infected mice until 50 days after infection. Untreated mice presented with a classic parasitemia curve peaking at day 8 after inoculation (Fig. 3), leading to the death of all (100%) the animals between 13 and 18 days post-infection (Table 1). All treatments were effective in reducing parasitemia compared with that in the untreated infected control ($P = 0.0007$) (Fig. 3A). The area under the parasitemia curve of the animals treated with the drug combinations was significantly reduced compared to those in the respective monotherapies (Fig. 3B).

Additionally, suboptimal doses of fex-SFN, BZ and NFX alone, although not inducing cure in animals, were able to reduce the parasitic load to levels undetectable by fresh blood tests and prevent mortality in all treated mice (Table 1). As the number of doses required to induce suppression of parasitemia is an indication of the activities of nitro compounds, we analysed the time to suppress the parasitemia among the groups. Treatment with fex-SFN suppressed parasite detection after 11.43 ± 4.58 doses; for BZ 50 mg kg^{-1}, 5.40 ± 4.96 doses were required and for NFX, 7.71 ± 4.89 doses. Interestingly, nitro drug associations led to a faster suppression of parasitemia than with monotherapies, with 1.28 ± 0.49 doses of BZ/fex-SFN and 1.37 ± 0.52 doses of NFX/fex-SFN comparable to standard treatment with BZ 100 mg kg^{-1} (1.43 ± 0.77 doses) (Table 1). After the end of the treatment, a natural parasitemia relapse was observed in all groups; however, this relapse was faster and in a larger number among those animals treated with monotherapies compared to those treated with combined therapy (Table 1).

To effectively verify the therapeutic potential of combined treatment using fex-SFN with BZ or NFX, we performed a stringent cure control protocol. The investigation of the reactivation of parasitism until 180 days after treatment was performed using blood/tissue PCR and immunosuppression with cyclophosphamide. In all monotherapy-treated mice, the parasite or its DNA was detected, evidencing therapeutic failure, as expected. Interestingly, when these suboptimal doses were administered in combination, they induced complete neutralization of parasitism in 83.3% (4 out of 6) of mice in the BZ/fex-SFN-treated group and in 75.0% (6 out of 8) of mice treated with NFX/fex-SFN, as these cure rates were higher than those observed with the reference treatment of BZ 100 mg kg^{-1} (57.1%, 4 out of 7) (Table 1). Likewise, the parasitic cardiac load confirmed the results, since in the group treated with NFX/fex-SFN, parasite DNA was detected in 25% of animals (2 out of 8, with 1.22 ± 0.31 parasites/25 μg of DNA). *Trypanosoma cruzi* DNA was not detected in the cardiac tissue of animals treated with BZ/fex-SFN (0 out of 6).

Interestingly, despite combining compounds that belong to similar therapeutic classes, we did not observe toxic effects resulting from the combined therapies. The analyses of the body weight of the mice during the treatment period demonstrated that, while 28.6% infected and untreated mice lost weight as a result of acute

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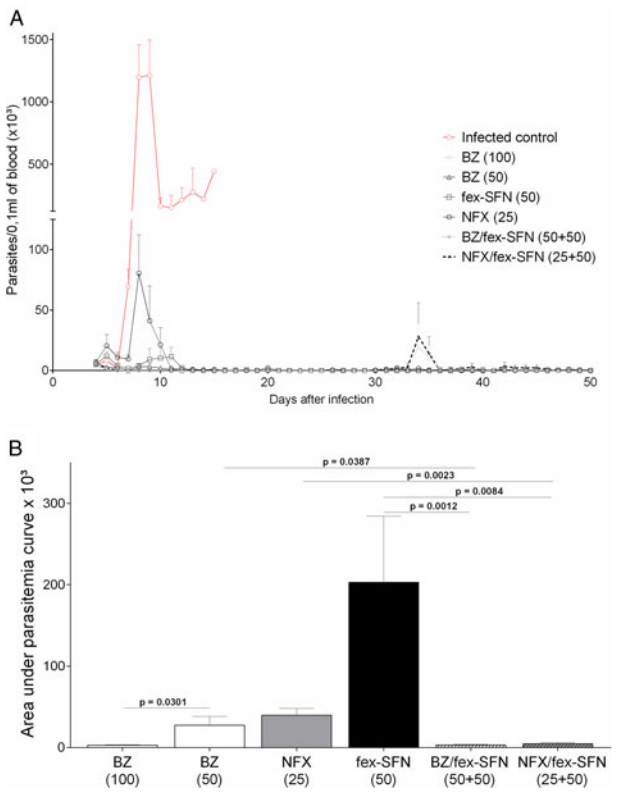

Fig. 3. Acute treatment with benznidazole (BZ), nifurtimox (NFX), the sulfone metabolite of fexinidazole (fex-SFN) or their combinations in mice infected with *Trypanosoma cruzi* Y strain. Parasitemia profile in mice treated for 20 days with BZ 100 or 25 mg kg^{-1}; NFX 25 mg kg^{-1}; fex-SFN 50 mg kg^{-1}; their combinations (BZ 50 mg kg^{-1} + fex-SFN 50 mg kg^{-1} and NFX 25 mg kg^{-1} + fex-SFN 50 mg kg^{-1}) and infected untreated control. Parasitemia was assessed during the treatment until 30 days post-treatment. (B) Area under the parasitemia curves of treated and infected mice.

infection, the treatments prevented this effect (Table 2). In addition, the weight gain observed in treated groups was significantly higher than in infected control, except for the NFX-treated groups. Similarly, all treatments were able to reduce liver enzyme levels in *T. cruzi* infection. Table 2 shows the levels of AST and ALT enzymes evaluated in the mice serum on the last day of treatment (and 15 days post-infection for infected and untreated mice). The BZ/fex-SFN-treated group reached AST and ALT levels similar to uninfected animals, and the NFX/fex-SFN-treated group showed ALT levels similar to uninfected controls. These reduced levels may reflect the decreased parasitic load in liver tissue, which would correspond to a decrease in tissue damage and safety of treatment.

Discussion

Incremental innovation to existing pharmaceutical products has been occurring in the form of supplementary approvals for new dosages, formulations and indications (Berndt *et al.*, 2006). This strategy has been fruitful in expanding the pharmacotherapeutic options of a number of diseases, and it is especially relevant to neglected ones. In this line and considering the scarce panel of new anti-*T. cruzi* molecules, alternative formulations (Leonardi *et al.*, 2013; Spósito *et al.*, 2017; Seremeta *et al.*, 2019; Mazzeti *et al.*, 2020; Rial *et al.*, 2020) and dosing regimens to reference medicines (Rial *et al.*, 2017; Kratz *et al.*, 2018; Mazzeti *et al.*, 2018; Perin *et al.*, 2020) have been explored in chemotherapy for Chagas disease. In parallel, the trypanocidal potential of another nitro drug has been revisited, allowing the identification of a promising candidate, such as fexinidazole, which has been recently included as a possible candidate to treat chronic *T. cruzi* infection (Patterson and Fairlamb, 2019; DNDi, 2020). Despite the safety concerns regarding this pharmacological class, nitro compounds have been explored with success as a source of a potential treatment for other kinetoplastid-related diseases (Patterson and Fairlamb, 2019), such as human African trypanosomiasis (Janssens and De Muynck, 1977; Torreele *et al.*, 2010; Eperon *et al.*, 2014; Tarral *et al.*, 2014; Mesu *et al.*, 2018) and leishmaniasis (Wyllie *et al.*, 2012; Koniordou *et al.*, 2017).

In this work, we demonstrated in a well-established preclinical model that combinations using fex-SFN with BZ or NFX are well tolerated and more effective than monotherapies at the same doses and even higher than standard BZ treatment, suggesting a positive interaction among the drugs. Although a number of studies have investigated the potential of combination therapy to increase the efficacy and reduce the toxicity of the reference treatments for Chagas disease (Araujo *et al.*, 2000; de Diniz *et al.*, 2013; Strauss *et al.*, 2013; Assíria Fontes Martins *et al.*, 2015; Providello *et al.*, 2018; Guedes-da-Silva *et al.*, 2019; Mazzeti *et al.*, 2019; Rocha Simões-Silva *et al.*, 2019), to the best of our knowledge, the interaction among nitro drugs has been sparsely studied (Cencig *et al.*, 2012).

First, the toxicity profile and nature of the interaction between BZ/fex-SFN and NFX/fex-SFN were assessed *in vitro*. The results confirmed that nitro drug derivatives are active on the micromolar scale (Bahia *et al.*, 2014; Moraes *et al.*, 2015; Mazzeti *et al.*, 2019) and showed that such combinations resulted in an additive effect in the absence of toxicity to host cells, with mean ΣFIC ranging from 0.57 to 0.84 (Fig. 1). Although there are no clear parameters to guide *in vitro* to *in vivo* progression related to anti-*T. cruzi* combinations (Machado *et al.*, 2020), we believe that is useful to move additive and non-toxic mixtures to evaluation in murine infection as a way to expand the preclinical data on combination therapy for Chagas disease.

In the next step, we performed an *in vivo* evaluation of combined treatments using suboptimal regimens – half of the standardized dose of each drug considering experimental chemotherapy (Filardi and Brener, 1987; Bahia *et al.*, 2012, 2014; Mazzeti *et al.*, 2018) as monotherapy and combined. These doses, while effective in suppressing the parasitemia, were unable to induce parasitological cure when used as monotherapy (Fig. 1, Table 1). On the other hand, when used concomitantly, they induced complete resolution of parasitism in 75.0% (NFX/fex-SFN) to 83.3% (BZ/fex-SFN) of infected mice (Table 1), suggesting a benefit from combined therapy. Classifying the nature of the interaction between drugs *in vivo* in the context of experimental Chagas disease remains a challenge. Unlike what is observed in *in vitro* experiments, where FIC and combination indexes can be calculated from dose-effect curves, *in vivo* analyses do not allow the estimation of parameters such as IC_{50} or IC_{90}. As a result, the effect of the combination was determined from the comparison with monotherapy and the reference treatment (Cencig *et al.*, 2012; Assíria Fontes Martins *et al.*, 2015; de Diniz *et al.*, 2018; Mazzeti *et al.*, 2019; Rocha Simões-Silva *et al.*, 2019).

Considering that the total dose of nitro compounds in the combination was equivalent or higher than the full standard dose (BZ 100 mg kg^{-1} or NFX 50 mg kg^{-1}), it would be reasonable to assume that the observed effects were the result of the total concentration of nitro drugs in the combination. However, the cure rates observed for combinations were higher than those obtained for the reference treatment with BZ (57.1%) (Table 1), demonstrating the greater effect of the drug combinations, particularly BZ/fex-SFN, compared to those with each drug alone. The mechanism of action of nitro drugs is not fully understood, but it involves similar pathways to BZ, NFX and fex-SFN. Further PK/PD analyses need to be performed to investigate the

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Table 1. Effect of treatment with nitro compounds (monotherapy or combination) in acute infection of mice by *Trypanosoma cruzi* Y strain[a]

Group (dose – mg kg^{-1})	Parasitemia clearance (days of treatment)	Parasitemia relapse (days)[b]	Positive FBE blood or PCR	Cure[c] (%)
Non-infected control	–	–	0/7	–
Infected control	0/7	–	7/7	0/7 (0%)
BZ (100)	7/7 (1.43 ± 0.77)	1/7 (4)	3/7	4/7 (57.1%)
BZ (50)	10/10 (5.40 ± 4.96)	7/10 (6.00 ± 8.70)	10/10	0/10 (0%)
NFX (25)	7/7 (7.71 ± 4.89)	4/7 (14.75 ± 2.87)	7/7	0/7 (0%)
fex-SFN (50)	7/7 (11.43 ± 4.58)	6/7 (14.75 ± 2.87)	7/7	0/7 (0%)
BZ/fex-SFN (50 + 50)	6/6 (1.28 ± 0.49)	0/6 (ND)	1/6	5/6 (83.3%)
NFX/fex-SFN (25 + 50)	8/8 (1.37 ± 0.52)	1/8 (7)	2/8	6/8 (75%)

BZ, benznidazole; NFX, nifurtimox; fex-SFN, sulfone metabolite of fexinidazole.
[a]Female Swiss (18–22 g) were inoculated with 5×10^3 trypomastigotes of Y *T. cruzi* strain. Treatments were started on the 4 days after infection, by gavage, for 20 consecutive days.
[b]Positive results in FBE (fresh blood examination) before immunosuppression with cyclophosphamide and PCR (polymerase chain reaction) assays, performed 30 days post-treatment. Parasitemia relapse after the end of treatment and the time (days) ± standard deviation.
[c]Cure rates based on negative results in fresh blood examination before and after immunosuppression with cyclophosphamide; blood qPCR (polymerase chain reaction) assays, performed 30 and 180 days post-treatment and tissue qPCR assays performed 180 days post-treatment.

Table 2. Toxicity of treatment with nitro compounds (monotherapy or combination) in acute infection of mice by *Trypanosoma cruzi*[1]

Group (dose – mg kg^{-1})	AST serum level (U l^{-1})[2]	ALT serum level (U l^{-1})[2]	Weight gain n/N (average of gain; %)	Mortality[4] n/N
Non-infected control	185.96 ± 61.45^{a}	72.58 ± 18.31^{a}	7/7 (24.24 ± 4.55)^{a}	0/7
Infected control	2596.40 ± 1181.46^{b}	585.81 ± 390.2^{b}	5/7 (6.75 ± 12.10)^{b}	7/7
BZ (100)	344.13 ± 85.56^{a,b}	89.51 ± 17.56^{a,b}	7/7 (21.69 ± 5.44)^{a}	0/7
fex-SFN (50)	340.66 ± 118.10^{a,b}	50.5 ± 14.7^{a,b}	7/7 (20.32 ± 11.15)	0/10
NFX (25)	380.8 ± 66.12^{a,b}	143.61 ± 74.12^{a,b}	7/7 (14.84 ± 6.76)^{b}	0/7
BZ/fex-SFN (50 + 50)	213.13 ± 30.92^{a}	56.43 ± 12.41^{a}	6/6 (19.38 ± 8.87)^{a}	0/6
NFX/fex-SFN (25 + 50)	380.00 ± 128.15^{a,b}	98.15 ± 51.84^{a}	8/8 (16.33 ± 10.35)	0/8

BZ, benznidazole; NFX, nifurtimox; fex-SFN, sulfone metabolite of fexinidazole.
[1]Female Swiss (18–22 g) were inoculated with 5×10^3 trypomastigotes of Y *T. cruzi* strain. Treatments were started on the 4 days after infection, by gavage, for 20 consecutive days.
[2]Levels of liver enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in the animals' serum on the last day of treatment. Levels of AST and ALT in serum of infected control animals were measured on the 15th day of infection.
a = statistical difference among infected control, $P < 0.05$.
b = statistical difference among non-infected control, $P < 0.05$.

molecular basis of the *in vivo* interaction among BZ/fex-SFN and NFX/fex-SFN. Interestingly, pharmacodynamically additive and synergistic drug combinations from molecules in the same pharmacological class and with the same mechanism of action have been demonstrated in the context of antiretroviral (Wertheimer and Morrison, 2002) and antibacterial chemotherapy (Jia *et al.*, 2009).

Herein, the data presented provide important information for the evaluation of reduced doses of nitro drug combinations in the treatment of human diseases in an attempt to increase efficacy. While one of the goals of combination therapy is to reduce drug doses and adverse effects, the potential for toxicity of the combined treatment in the host needs to be monitored to ensure that there is no potentiation of the adverse effects. In preclinical assays, an important aspect to note is whether the treatment induces liver damage (Amacher, 1998). Likewise, our data showed that the level of liver enzymes (AST and ALT) detected in the serum of the animals treated with nitro drug combinations on the last day of treatment was significantly lower than those in the serum of the infected control animals and close to the level found in the serum of uninfected and untreated animals. In addition, this may be a reflection of the decrease in the parasitic load in hepatic tissue that would correspond to a decrease in tissue damage (Novaes *et al.*, 2015). In the same way, weight gain and mortality rates of infected and treated mice demonstrated the benefit of treatment in the absence of apparent toxicity.

Altogether, our results indicated that the combination of fex-SFN with the first-line drugs BZ or NFX is a promising alternative for the treatment of Chagas disease. Considering that the toxicological profile of this pharmacological class is related to higher doses or prolonged regimens (Pérez-Molina and Molina, 2018), the positive interaction observed could allow the reduction of the doses of each compound without changing or increasing the effectiveness of the treatment. Further experiments using different *T. cruzi* strains, infection phases and PK/PD profiles of monotherapy *vs* combination therapy need to be carried out to validate this strategy. Although many answers are needed, this study is the starting point to explore the potential effects of nitro drug combinations in preclinical models of *T. cruzi* infection and other kinetoplastid-related diseases.

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Ethical standards. The Ethics Committee in Animal Research at the Federal University of Ouro Preto, Minas Gerais, Brazil (UFOP), approved *in vivo* procedures and experimental conditions (number 2009/17).

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