

# Activity profile of two 5-nitroindazole derivatives over the moderately drug-resistant *Trypanosoma cruzi* Y strain (DTU TcII): *in vitro* and *in vivo* studies

## Research Article

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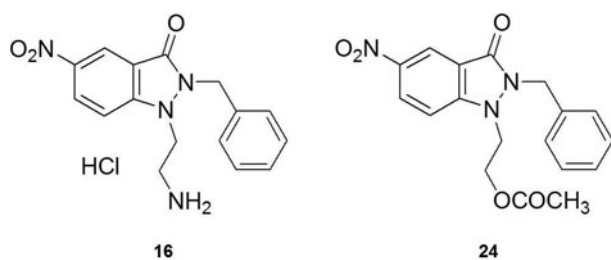
### Abstract

In previous studies, we have identified several families of 5-nitroindazole derivatives as promising antichagasic prototypes. Among them, 1-(2-aminoethyl)-2-benzyl-5-nitro-1,2-dihydro-3H-indazol-3-one, (hydrochloride) and 1-(2-acetoxyethyl)-2-benzyl-5-nitro-1,2-dihydro-3H-indazol-3-one (compounds 16 and 24, respectively) have recently shown outstanding activity *in vitro* over the drug-sensitive *Trypanosoma cruzi* CL strain (DTU TcVI). Here, we explored the activity of these derivatives against the moderately drug-resistant Y strain (DTU TcII), *in vitro* and *in vivo*. The outcomes confirmed their activity over replicative forms, showing IC<sub>50</sub> values of 0.49 (16) and 5.75 μM (24) towards epimastigotes, 0.41 (16) and 1.17 μM (24) against intracellular amastigotes. These results, supported by the lack of toxicity on cardiac cells, led to better selectivities than benznidazole (BZ). Otherwise, they were not as active as BZ *in vitro* against the non-replicative form of the parasite, i.e. bloodstream trypomastigotes. *In vivo*, acute toxicity assays revealed the absence of toxic events when administered to mice. Moreover, different therapeutic schemes pointed to their capability for decreasing the parasitaemia of *T. cruzi* Y acute infected mice, reaching up to 60% of reduction at the peak day as monotherapy (16), 79.24 and 91.11% when 16 and 24 were co-administered with BZ. These combined therapies had also a positive impact over the mortality, yielding survivals of 83.33 and 66.67%, respectively, while untreated animals reached a cumulative mortality of 100%. These findings confirm the 5-nitroindazole scaffold as a putative prototype for developing novel drugs potentially applicable to the treatment of Chagas disease and introduce their suitability to act in combination with the reference drug.

### Introduction

Neglected tropical diseases (NTDs) are a group of 20 ailments that, despite showing high prevalence in different developing areas around the world, do not receive enough attention to be completely controlled and eliminated (WHO, 2017). Among the parasitic NTDs, Chagas disease (CD, aka American trypanosomiasis), caused by the intracellular protozoan pathogen *Trypanosoma cruzi*, is a vector-borne infection that is naturally transmitted by the contaminated feces of blood-sucking triatomine bugs. In fact, the parasite is mainly transmitted by these reduviid vectors along the 21 Latin American countries where CD is currently endemic (WHO, 2017). However, this classical vectorial route is not the only mechanism of *T. cruzi* transmission: outbreaks of orally-acquired infections, associated either to contaminated food or beverage, have also been reported in several regions of the endemic area (e.g. the Amazon Basin) (Shikanai-Yasuda and Carvalho, 2012) and other alternative routes, such as the iatrogenic and the congenital one, have significantly contributed to both the spreading and the introduction of the parasitic disease in non-endemic countries, like Spain (Pinazo and Gascón, 2015). Currently, the epidemiological scenario of CD comprises about 6–7 million of infected individuals and over 75 million people living at risk worldwide (WHO, 2017).

As in other NTDs caused by kinetoplastid parasites (i.e. sleeping sickness and leishmaniasis), the necessity of identifying novel treatment strategies for combating *T. cruzi* infection is still a reality (Patterson and Wylie, 2014; Scarim *et al.*, 2018). Indeed, CD chemotherapy is currently based on two old nitroaromatic drugs: the 2-nitroimidazole benznidazole (BZ) and the 5-nitrofuran nifurtimox, which show important disadvantages related to their effectiveness in the later chronic stage of the disease, besides the wide range of side-effects they induce in patients (Urbina, 2018; Ribeiro *et al.*, 2020). Presently, more than five decades after the introduction of these two medicines, a promising drug candidate for CD therapy is fexinidazole,



**Fig. 1.** Chemical structures of the two 5-nitroindazole derivatives assayed: 1-(2-aminoethyl)-2-benzyl-5-nitro-1,2-dihydro-3H-indazol-3-one, hydrochloride (16) and 1-(2-acetoxyethyl)-2-benzyl-5-nitro-1,2-dihydro-3H-indazol-3-one (24).

another nitroheterocyclic compound that is the first all-oral treatment approved for both stages of *Trypanosoma brucei gambiense* human infection, the most common form of sleeping sickness (Patterson and Wyllie, 2014; WHO, 2019). According to these findings, it is noteworthy to point out that nitroheterocycles are still considered as a tempting group of molecules to be explored for the identification of novel CD treatments (Patterson and Fairlamb, 2018). Several families of synthetic compounds that contain nitro groups in their scaffolds and also commercialized drugs identified by repurposing strategies, such as the 5-nitroimidazole metronidazole, have been recently proposed as putative anti-*T. cruzi* agents, according to the promising activity they achieved in different experimental models of the disease, both *in vitro* and *in vivo* (Simões-Silva *et al.*, 2017; Thompson *et al.*, 2017; Almeida *et al.*, 2018; Arias *et al.*, 2018; Leite *et al.*, 2018; Palace-Berl *et al.*, 2018; Chaves e Mello *et al.*, 2020). Nevertheless, toxicity issues are usually attributed to this kind of compounds (Patterson and Wyllie, 2014) and thus, it is mandatory to discard any potential risk associated to the presence of a nitro group in the antichagasic prototype.

In this context, our research group has identified various trypanocidal agents among several families of 5-nitroindazole derivatives. The most promising molecules of a synthetic series of 1,2-disubstituted 5-nitroindazolinones showed remarkable activity over the replicative forms (i.e. extracellular epimastigotes and intracellular amastigotes) of *T. cruzi* strains that are representative of different discrete typing units (DTUs): the drug-susceptible CL and Tulahuen (both, DTU TcVI) and the moderately drug-resistant Y (DTU TcII). These prototypes also registered no toxic effects on different mammalian cells, such as J774 macrophages, L929 fibroblasts and primary cultures of cardiac cells (CC) (Vega *et al.*, 2012; Fonseca-Berzal *et al.*, 2015). Among them, 2-benzyl derivatives were identified as the best anti-*T. cruzi* compounds of this synthetic series (Vega *et al.*, 2012). Several of these 1-substituted 2-benzyl-5-nitroindazolinones also induced important reductions in the parasitaemia levels of *T. cruzi* acute infected mice (Fonseca-Berzal *et al.*, 2014). According to these results, other series of 5-nitroindazole derivatives were subsequently synthesized by our research group, all of them supporting a benzyl moiety at the N-2 position of the indazole scaffold, i.e. 3-alkoxy-2-benzyl-5-nitro-2H-indazoles and 1-alkyl-2-benzyl-5-nitroindazol-3-ones. Among these two series, we have recently identified several molecules that show great selectivity against the causative agent of CD (Fonseca-Berzal *et al.*, 2016a, 2018).

In the present study, we evaluated the activity profile of two of the most selective compounds of the aforementioned series of 1-alkyl-2-benzyl-5-nitroindazol-3-ones: 1-(2-aminoethyl) derivative 16 and 1-(2-acetoxyethyl) derivative 24 (Fig. 1), against *T. cruzi* parasites of Y strain (DTU TcII). Aiming to further explore the trypanocidal spectrum of these 5-nitroindazole derivatives, we assayed them *in vitro* over the three main parasite forms [i.e. epimastigotes, bloodstream trypomastigotes (BT) and intracellular

amastigotes] of a *T. cruzi* strain that naturally shows moderate resistance to nitroderivatives (Romanha *et al.*, 2010; Soeiro *et al.*, 2013). Furthermore, these molecules were moved to *in vivo* mouse models of both acute toxicity and *T. cruzi* infection, to assess their effectiveness when administered through different routes, either as monotherapy or combined with the reference drug BZ.

## Material and methods

### Compounds

The synthesis of the two 5-nitroindazole derivatives studied in the present work (Fig. 1) was previously described (Fonseca-Berzal *et al.*, 2016a, 2018). The numbering initially used for the designation of these molecules in their respective references has been maintained in the present paper, as follows: 1-(2-aminoethyl)-2-benzyl-5-nitro-1,2-dihydro-3H-indazol-3-one (hydrochloride), compound 16 (Fonseca-Berzal *et al.*, 2018) and 1-(2-acetoxyethyl)-2-benzyl-5-nitro-1,2-dihydro-3H-indazol-3-one, compound 24 (Fonseca-Berzal *et al.*, 2016a). For all the *in vitro* assays, stock solutions of 16, 24 and BZ – purchased from LAFEPE, Laboratório Farmacêutico do Estado de Pernambuco, Brazil – were prepared in dimethyl sulfoxide (DMSO) and extemporaneously added to the cultures in a final concentration of the solvent lower than 1% (v/v), which has no toxic effect on cultures of mammalian cells and parasites (Fonseca-Berzal *et al.*, 2015). Regarding the *in vivo* experiments, compounds 16 and 24 were diluted in either 10% DMSO (v/v) or 20% Trappsol (w/v), both prepared in distilled and sterile water. For the administration of BZ, the reference drug was always dissolved in distilled and sterile water supplemented with 3% Tween 80 (v/v). All the final concentrations used for preparing these vehicles do not cause any detectable toxic effect (i.e. behaviour and clinical parameters) on mice (da Silva *et al.*, 2017).

### Mammalian cells

Primary cultures of embryonic CC were obtained from Swiss Webster mice as described in Meirelles *et al.* (1986). Purified CC cultures were incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and sustained in Dulbecco's Modified Eagle's Medium (DMEM) without phenol red, supplemented with 2.5 mM CaCl<sub>2</sub>, 1 mM L-glutamine, 2% chicken embryo extract and 5% fetal bovine serum (FBS) previously inactivated by heating at 56°C for 30 min (Fonseca-Berzal *et al.*, 2015).

### Parasites

In this study, all the experiments were performed using *T. cruzi* Y strain (DTU TcII), which was initially isolated from an acute human case in Brazil and characterized by Silva and Nussenzweig (1953). Log-phase cultures of epimastigotes were continuously maintained at 28°C in Liver Infusion Tryptose (LIT) medium, supplemented as described in Vega *et al.* (2012). BT were obtained from acute infected Swiss Webster mice by performing heart puncture at the parasitaemia peak day and after being purified, BT were suspended in Roswell Park Memorial Institute (RPMI) medium supplemented with 5% heat-inactivated FBS (Fonseca-Berzal *et al.*, 2015). Intracellular forms were obtained by the infection of CC with BT in a 1:10 ratio (cell:parasite), as previously depicted (Fonseca-Berzal *et al.*, 2015; da Silva *et al.*, 2017).

### Animals

The use of outbred stock Swiss Webster male mice and the experimental procedures were in accordance with the Brazilian Law

11.794/2008 and the regulations of the National Council for the Control of Animal Experimentation (CONCEA). The mice were housed at a maximum of six individuals per cage, kept in a specific-pathogen-free (SPF) room at 20–22°C and 50–60% humidity under a 12/12 h light/dark cycle, and provided with sterilized water and chow *ad libitum*. The animals were adapted to these conditions for 1 week before starting the experiments. All animal experimental procedures were performed following the license L-038/2017 approved by the Ethics Committee for Animal Use of Instituto Oswaldo Cruz (CEUA/IOC).

### In vitro experiments

#### Unspecific cytotoxicity on CC cultures

The toxicity profile of compounds 16 and 24 was determined over CC, by applying a colorimetric method in the presence of the redox indicator Prestoblue®. The assay was performed in 96-well plates previously coated with 0.01% of gelatin (Meirelles *et al.*, 1986), and 100 µL of DMEM containing  $6 \times 10^4$  cells were seeded per well. These plates were incubated overnight at 37°C with 5% CO<sub>2</sub> and then, the medium was replaced by serial solutions of each compound in fresh DMEM. Cultures of CC were treated for 24 and 48 h under the same conditions of temperature and humidity, and controls of non-treated and BZ-treated cells included in every plate. After each time point, both CC morphology and contractibility were examined by light microscopy, and cell viability monitored by adding 10% Prestoblue® (v/v). The plates were finally incubated for 5 h under the conditions aforementioned and absorbance read at 570 and 600 nm. Each assay was performed separately three times ( $n = 3$ ) and LC<sub>50</sub> (i.e. concentration that induces 50% of cell lethality) values determined after both 24 and 48 h of compound exposure, by plotting drug concentrations vs percentages of cytotoxicity (%C) (Fonseca-Berzal *et al.*, 2015; da Silva *et al.*, 2017).

#### Epimastigote susceptibility assay

The activity against epimastigotes was determined by applying the spectrofluorimetric method standardized by Rolón *et al.* (2006). In a 96-well microplate, cultures of log-phase epimastigotes in LIT medium ( $3 \times 10^6$  epimastigotes mL<sup>-1</sup>) were seeded by adding 200 µL per well. The parasites were exposed to serial solutions of compounds 16 and 24 for 48 h at 28°C, and controls of non-treated and BZ-treated epimastigotes included in each assay. Finally, 10% resazurin solution (3 mM in PBS, pH 7.0) was added (v/v) and after incubating 5 h at 28°C, fluorescent intensity read at 535 (excitation) and 590 nm (emission) wavelengths. Each assay was run similarly for three ( $n = 3$ ) and both IC<sub>50</sub> and IC<sub>90</sub> (i.e. concentration that inhibits 50 and 90% of epimastigote growth, respectively) estimated by plotting drug concentrations vs percentages of parasite growth inhibition (%PGI). For each compound, selectivity index (SI) on epimastigotes was calculated, as follows:  $SI_{EPI} = LC_{50}/IC_{50}$ , where LC<sub>50</sub> corresponds to the value obtained after 48 h of CC treatment.

#### BT susceptibility assay

This assay was performed following the methodology described in previous works (Fonseca-Berzal *et al.*, 2016b; da Silva *et al.*, 2017). Briefly, a suspension containing  $5 \times 10^6$  BT mL<sup>-1</sup> in RPMI medium was distributed in a 96-well microplate by seeding 200 µL per well. Then, the parasites were incubated within serial solutions of compounds 16 and 24, for 24 h at 37°C and 5% CO<sub>2</sub>. Controls of non-treated and BZ-treated BT were included in each experiment. Finally, parasite death rates were determined with a light microscope, by directly counting the number of live BT in a Neubauer chamber. Each assay was run in similar conditions at least two times ( $n \geq 2$ ) and both EC<sub>50</sub> and EC<sub>90</sub> (i.e.

concentration that provokes 50 and 90% of parasite death, respectively) estimated by plotting drug concentrations vs percentages of parasite death (%PD). For each compound, SI on BT was also calculated, as follows:  $SI_{BT} = LC_{50}/EC_{50}$ , where LC<sub>50</sub> corresponds to the value obtained after 24 h of CC treatment.

#### Intracellular amastigote susceptibility assay

For this assay, 100,000 CC per well were seeded in a 24-well plate provided with rounded coverslips previously coated with 0.01% of gelatin and then, maintained in DMEM for 24 h at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. Afterwards, CC were infected with Y strain BT in a ratio 1:10 (cell:parasite) and this interaction was maintained for 24 h at 37°C with 5% CO<sub>2</sub>. These infected CC were washed to remove non-internalized parasites and then, incubated for 48 h within compounds 16 and 24 serially diluted in fresh medium. Controls of untreated infected CC were included in all the plates and BZ was tested in parallel. Finally, the infected CC were fixed with Bouin and stained with Giemsa and the trypanocidal activity determined by means of light microscopy, scoring the percentage of infected CC and the mean number of parasites per infected CC to estimate the endocytic index (EI). Each assay was run similarly at least two times ( $n \geq 2$ ) and both IC<sub>50</sub> and IC<sub>90</sub> (i.e. concentration that inhibits 50 and 90% of the EI, respectively) obtained by plotting drug concentrations vs percentages of EI inhibition (%EII). The respective SI on amastigotes was also estimated:  $SI_{AMA} = LC_{50}/IC_{50}$ , where LC<sub>50</sub> is the value obtained after 48 h of CC treatment (Fonseca-Berzal *et al.*, 2015; da Silva *et al.*, 2017).

### In vivo experiments

#### Acute toxicity in mice

Acute toxicity levels of derivatives 16 and 24 were determined in male Swiss Webster mice weighing  $28 \pm 4$  g. Escalating doses of both compounds were administered every 2 h to groups of two mice, either orally (p.o.) or intraperitoneally (ip), until reaching a final cumulative dose of 200 mg kg<sup>-1</sup>. A group of mice treated with the vehicle alone was included in each experiment, as control. Conform to the Organization for Economic Cooperation and Development (OECD) guidelines, animal acute toxicity was evaluated every 2 h for a 24 h period, and also 48 h post-treatment, in order to detect any toxic and/or sub-toxic (i.e. mobility, motor locomotion, tremors, convulsions or pain signals, such as prostration and both facial and corporal expression) symptoms. Moreover, chow and water consumption was observed and body weight monitored. The possible cardiotoxicity was evaluated by exploring the integrity of the electrical conduction system by electrocardiographic (ECG) traces analysis. After the complete observation period of 48 h, NOAEL (*no-observed-adverse-effect level*) was determined. At this endpoint time, the animals were euthanized and their organs (i.e. kidney, liver, heart and spleen) inspected to look for morphological abnormalities. Serum samples were also collected for conducting biochemical analyses in ICTB/FIOCRUZ, to detect possible alterations in the plasmatic levels of both alanine (ALT) and aspartate (AST) aminotransferases, creatinine and creatine kinase (CK), which are indicative markers of hepatic, renal and muscular injury, respectively. Each experiment was performed twice ( $n = 2$ ) in similar conditions (Timm *et al.*, 2014; da Silva *et al.*, 2017; Simões-Silva *et al.*, 2017).

#### Effectiveness on a murine model of acute infection

Male Swiss Webster mice with an average weight of  $22 \pm 2$  g were infected with  $10^4$  BT forms of *T. cruzi* Y strain inoculated *via ip*. In all the assays, only those animals with positive parasitaemia at 5th day post-infection (dpi), which corresponds to the

parasitaemia onset in this model, composed the experimental groups. In a first study ( $n = 5$  mice/group), for the purpose of evaluating the effectiveness of compounds 16 and 24 when prepared by using two different vehicles (i.e. 10% DMSO or 20% Trappsol) and administered to mice either p.o. or ip, the animals were randomly divided in groups, as follows: (i) non-infected and untreated (i.e. negative control), (ii) infected and untreated, receiving the vehicle alone (i.e. control of infection), (iii) infected and treated p.o. with 25 mg kg<sup>-1</sup> of 16 in 10% DMSO, (iv) infected and treated ip with 25 mg kg<sup>-1</sup> of 16 in 10% DMSO, (v) infected and treated p.o. with 25 mg kg<sup>-1</sup> of 16 in 20% Trappsol, (vi) infected and treated ip with 25 mg kg<sup>-1</sup> of 16 in 20% Trappsol, (vii) infected and treated p.o. with 25 mg kg<sup>-1</sup> of 24 in 10% DMSO, (viii) infected and treated ip with 25 mg kg<sup>-1</sup> of 24 in 10% DMSO, (ix) infected and treated p.o. with 25 mg kg<sup>-1</sup> of 24 in 20% Trappsol, (x) infected and treated ip with 25 mg kg<sup>-1</sup> of 24 in 20% Trappsol, (xi) infected and treated p.o. with a combination of 16 in 10% DMSO and BZ (both, 25 mg kg<sup>-1</sup>) and (xii) infected and treated p.o. with 25 mg kg<sup>-1</sup> of BZ. For each therapeutic scheme, the mice received two unique doses of 0.1 mL at 5th and 8th dpi. A second study ( $n = 6$  mice/group), was also carried out, in which the mice were randomly distributed in the following groups: (i) non-infected and untreated (i.e. negative control), (ii) infected and untreated, receiving the vehicle alone (i.e. control of infection), (iii) infected and treated with 25 mg kg<sup>-1</sup> of 16, (iv) infected and treated with 25 mg kg<sup>-1</sup> of 24, (v) infected and treated with a combination of 16 and BZ (both, 25 mg kg<sup>-1</sup>), (vi) infected and treated with a combination of 24 and BZ (both, 25 mg kg<sup>-1</sup>) and (vii) infected and treated with 25 mg kg<sup>-1</sup> of BZ. In this case, compounds were always diluted in 10% DMSO and given to the mice in five consecutive daily doses of 0.1 mL p.o., beginning at 5th dpi. In both experiments and for evaluating the effectiveness of the aforementioned treatments, parasitaemia levels were individually checked, by directly counting the number of BT in 5  $\mu$ L of blood taken from the tail of each mouse, following the method described by Brener (1962). Moreover, body weights were controlled weekly (i.e. ponderal curve analysis) and mortality rates monitored daily until the 30th day post-treatment and expressed as the percentage of cumulative mortality (%CM) (de Araújo *et al.*, 2014; Timm *et al.*, 2014; da Silva *et al.*, 2017; Simões-Silva *et al.*, 2017). Finally, the cardiac function of those surviving mice that progressed to the chronic stage of the infection was explored by the evaluation of ECG parameters: frequency, PR, QRS and QT intervals, as described in Campos *et al.* (2016). In order to preserve animal welfare, daily observation was supervised by a PhD veterinary doctor aiming to avoid any suffering and pain as a result of parasite infection and/or compound administration. The mice were humanely euthanized whenever early endpoints of animal suffering became apparent, such as motor disturbance, exploratory activity and/or moribund condition.

### Statistical analysis

SPSS Statistics Software (version 22, IBM) was used for the statistical analysis. The non-parametric Mann–Whitney  $U$  test was applied for comparing the activity and cytotoxic profile *in vitro* of both 5-nitroindazole derivatives 16 and 24 with those of the reference drug BZ. The same test was run for analysing the trypanocidal activity of these compounds among representative strains of DTUs TcII and TcVI. Moreover, the non-parametric Kruskal–Wallis test was used for conducting comparative studies among schemes assessed in the acute toxicity model. Regarding the effectiveness *in vivo*, the impact of these treatments over parasitaemia levels, body weight and cardiac function of *T. cruzi*-infected mice was analysed by applying one-way ANOVA, with the

subsequent post-hoc analysis. In all the studies,  $P < 0.05$  was considered statistically significant.

## Results

### In vitro experiments

#### Unspecific cytotoxicity on CC cultures

None of these 5-nitroindazole derivatives induced important cytotoxic effects over primary cultures of CC. After both 24 and 48 h of compounds exposure, neither morphology nor contractibility affectation was observed: only derivative 16 seemed to be slightly cytotoxic at the highest concentration tested (i.e. 200  $\mu$ M) after 48 h of incubation (%C = 46.45%). In fact, 16 and 24 showed LC<sub>50</sub> values higher than 200  $\mu$ M at these two time points and cytotoxic profiles comparable to the one exhibited by the reference drug BZ (Supplementary Fig. S1).

#### Activity against epimastigotes

The activity of derivatives 16 and 24 over the extracellular and replicative form of *T. cruzi* is depicted in both Fig. 2 and Table 1. These compounds were considerably more active on Y strain epimastigotes than BZ ( $P < 0.05$ ), showing IC<sub>50</sub> values lower than 10  $\mu$ M and higher selectivity than the reference drug (Table 1). As it can be seen in Fig. 2, none of them achieved the IC<sub>90</sub> value at the higher concentration tested. However, at 20  $\mu$ M both derivatives inhibited epimastigote growth in a percentage higher than that of the reference drug, showing %PGI of 87.29, 83.36 and 63.84% (i.e. derivatives 16, 24 and BZ, respectively). Among these two molecules, compound 16 displayed the most outstanding trypanocidal profile, with IC<sub>50</sub> = 0.49  $\mu$ M ( $P < 0.05$ ) and SI<sub>EPI</sub> > 408.16.

#### Activity against BT

Regarding the activity on BT (Fig. 2 and Table 1) after 24 h of incubation at 37°C, compounds 16 and 24 did not have any impact over the extracellular and non-replicative form of *T. cruzi*, being both molecules less effective than BZ (EC<sub>50</sub> = 7.43  $\mu$ M). Indeed, none of these 5-nitroindazole derivatives reached the EC<sub>50</sub> at the concentrations tested (EC<sub>50</sub> > 50  $\mu$ M) and thus, their SI<sub>BT</sub> could not be determined (ND).

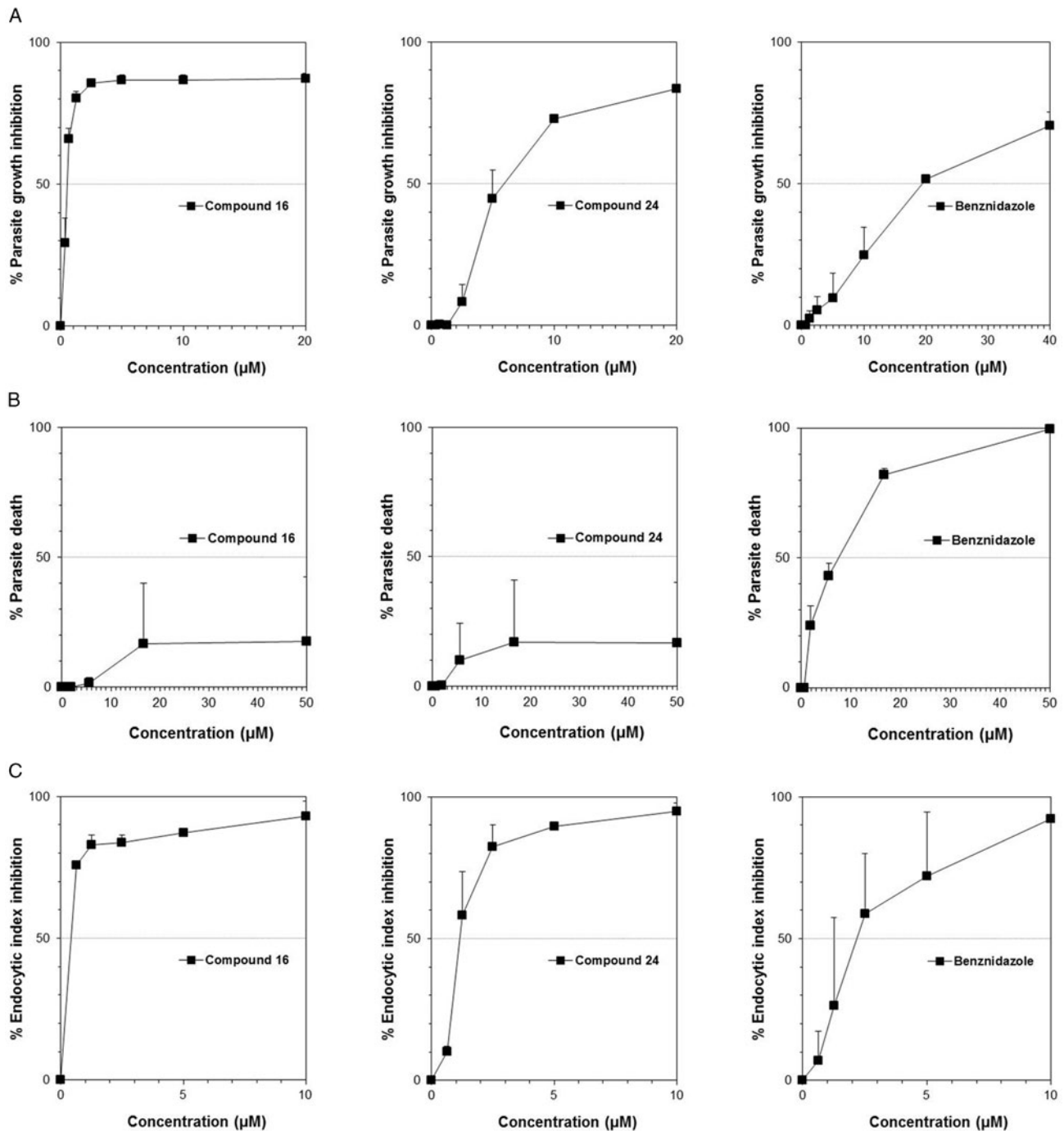
#### Activity against intracellular amastigotes

Concerning the activity over intracellular amastigotes, both 5-nitroindazole derivatives exhibited outstanding trypanocidal profiles against the intracellular and replicative form of *T. cruzi* (Fig. 2 and Table 1). Certainly, these compounds were more active than the reference drug BZ, being derivative 16 the most promising one: IC<sub>50</sub> = 0.41  $\mu$ M ( $P < 0.05$ ), SI<sub>AMA</sub> > 487.80.

### In vivo experiments

#### Acute toxicity in mice

As Supplementary Table S1 reflects, neither death nor apparent toxic effects were found in mice by clinical observation, as a consequence of the administration of derivatives 16 and 24. Certainly, compound 16 showed NOAEL > 200 mg kg<sup>-1</sup> when it was given either p.o. or ip, and the same toxicity profile was observed with the oral administration of compound 24. However, some solubility problems were detected when this derivative was prepared in doses >100 mg kg<sup>-1</sup>. Accordingly, the administration of compound 24 *via* ip was interrupted after giving the third dose (i.e. 50 mg kg<sup>-1</sup>), reaching a cumulative dose of 100 mg kg<sup>-1</sup> and thus, considering NOAEL > 100 mg kg<sup>-1</sup>. Furthermore, those mice that every 2 h received 0.1 mL of the vehicle alone



**Fig. 2.** Trypanocidal profile of 5-nitroindazole derivatives 16 (left), 24 (middle) and BZ (right) against Y strain epimastigotes (A), bloodstream trypomastigotes (B) and intracellular amastigotes (C).

(both, p.o. and ip) did not exhibit detectable side-effects too (data not shown).

Regarding the biochemical analysis, plasmatic levels of ALT, AST, creatinine and CK did not show significant alterations after the administration of derivatives 16 and 24 (Supplementary Table S2), pointing to the absence of any hepatic, renal and cardiac dysfunction, respectively. Similarly, those control mice that received the vehicle alone maintained these parameters unaltered (data not shown).

Finally, at the endpoint time (i.e. 48 h post-treatment), loss of body weight (Fig. 3A) and morphological alterations in kidney, liver, heart and spleen (Fig. 3B) were not registered in any group. Moreover, all these treated mice showed ECG profiles with frequencies, PR, QRS and QT intervals similar to those of

the non-treated group, which indicated the absence of abnormalities in the cardiac function during the observation period. In fact, significant differences were not noticed when comparing the ECG profiles obtained 48 h post-treatment with those recorded before the administration of these compounds (Fig. 3C). As it can be seen in Fig. 3, all the parameters studied in the groups treated with derivatives 16 and 24, administered either p.o. or ip, were similar to those of the respective control group that received the vehicle alone.

#### *Effectiveness on a murine model of acute infection*

Since none of these 5-nitroindazole derivatives induced acute toxicity effects when administered to mice either p.o. or ip, different therapeutic schemes of both 16 and 24 were tested in a

**Table 1.** Activity of 5-nitroindazole derivatives 16 and 24 against epimastigotes, bloodstream trypomastigotes and intracellular amastigotes of *T. cruzi* Y strain, expressed as IC<sub>50</sub> and IC<sub>90</sub> (EC<sub>50</sub> and EC<sub>90</sub> for bloodstream trypomastigotes)

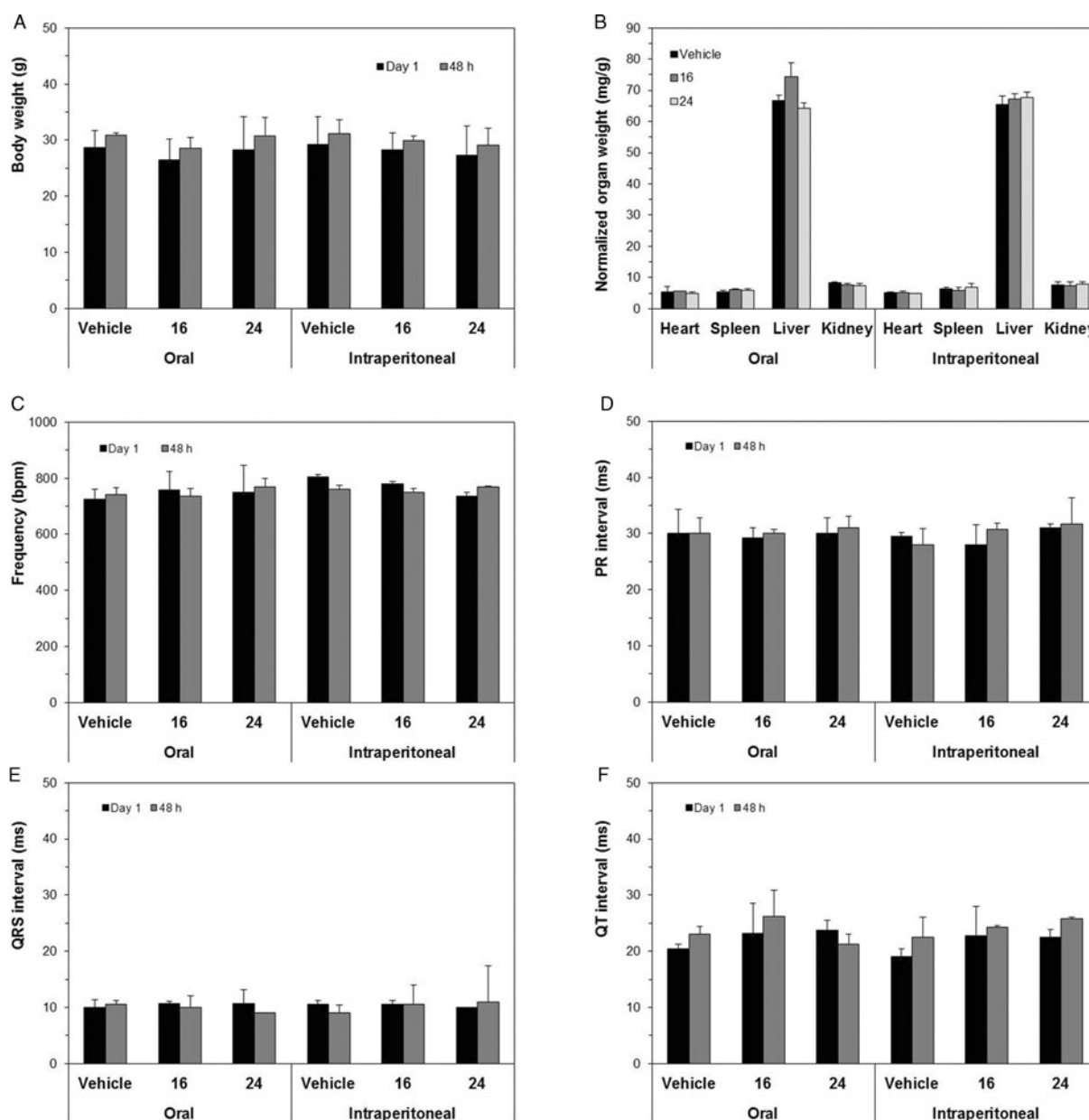
| Compound | Epimastigotes – 48 h at 28°C |                       |                   | Bloodstream trypomastigotes – 24 h at 37°C |                       |                  | Intracellular amastigotes – 48 h at 37°C |                       |                   |
|----------|------------------------------|-----------------------|-------------------|--|-----------------------|------------------|--|-----------------------|-------------------|
|          | IC <sub>50</sub> (μM)        | IC <sub>90</sub> (μM) | SI <sub>EPI</sub> | EC <sub>50</sub> (μM)                      | EC <sub>90</sub> (μM) | SI <sub>BT</sub> | IC <sub>50</sub> (μM)                    | IC <sub>90</sub> (μM) | SI <sub>AMA</sub> |
| 16       | 0.49 ± 0.02*                 | >20                   | >408.16           | >50  | >50                   | ND               | 0.41 ± 0.00*                             | 7.13 ± 0.41           | >487.80           |
| 24       | 5.75 ± 1.23*                 | >20                   | >34.78            | >50  | >50                   | ND               | 1.17 ± 0.17                              | 5.32 ± 0.59           | >170.94           |
| BZ       | 19.28 ± 0.49                 | >40                   | >10.37            | 7.43 ± 1.00                                | 31.64 ± 2.70          | >26.92           | 2.18 ± 0.82                              | 9.39 ± 3.54           | >91.74            |

Results are expressed as the mean ± s.d. of at least two independent assays ( $n \geq 2$ ). Selectivity indexes (SI) on each parasite form are also estimated.

ND, not determined.

\*Significant differences ( $P < 0.05$ ) with BZ, Mann-Whitney *U* test.

#Significant differences ( $P < 0.05$ ) with the activity over a DTU TcV1 strain (see Fonseca-Berzal *et al.*, 2016a, 2018), Mann-Whitney *U* test.

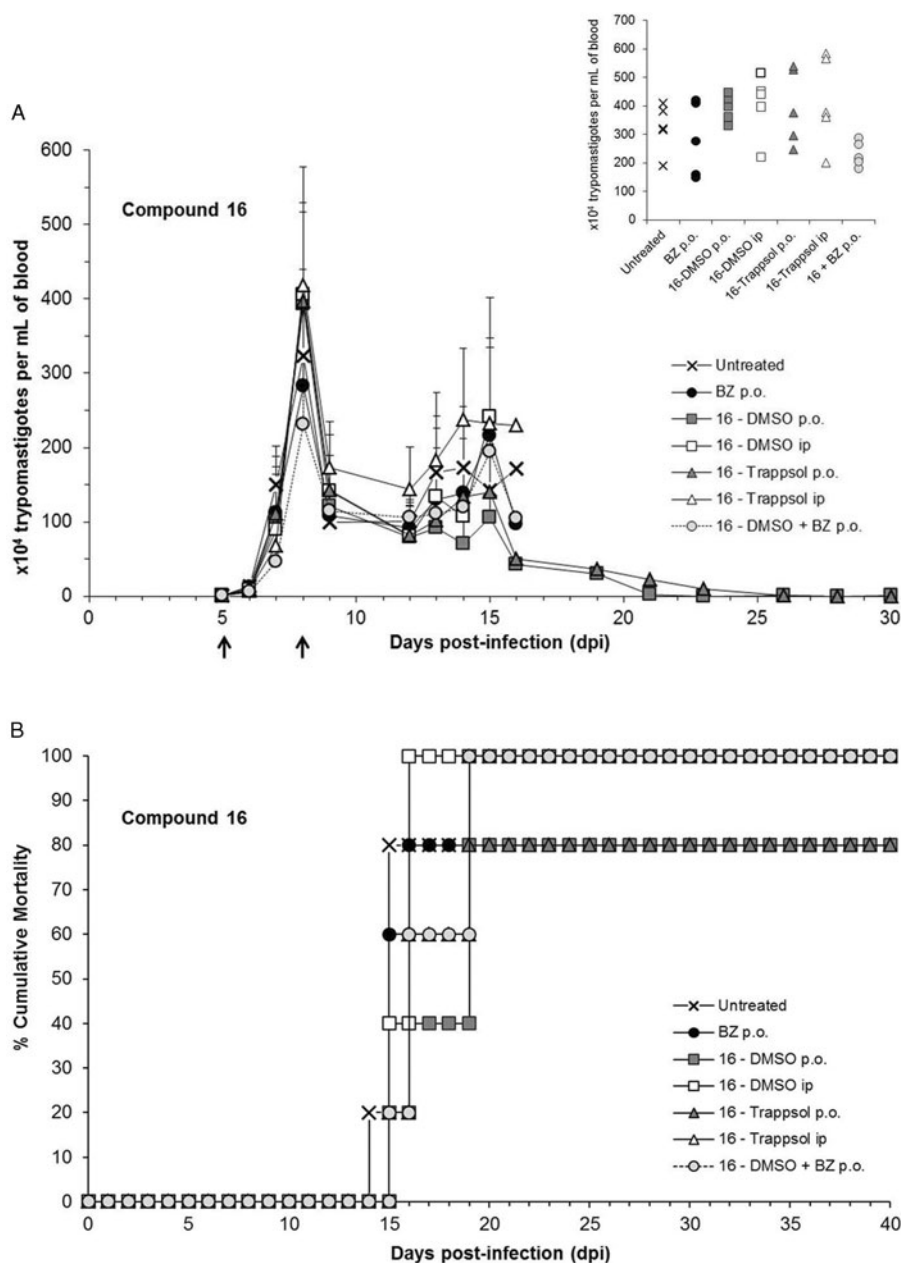


**Fig. 3.** Variations in body weight (A), normalized organ weight (B) and electrocardiographic parameters (C) of male Swiss Webster mice 48 h after concluding the treatments with 5-nitroindazole derivatives 16 and 24. Bars represent the mean ± s.d. of the results obtained in two independent assays ( $n = 2$ ). \*Significant differences ( $P < 0.05$ ) with the respective control group, Kruskal-Wallis. #Significant differences ( $P < 0.05$ ) with the parameters observed at day 1, Kruskal-Wallis.

mouse model of acute infection caused by *T. cruzi*. In a first experiment, the infected mice were treated with two unique doses of 25 mg kg<sup>-1</sup>, which were administered at 5th (i.e. parasitaemia onset) and 8th (i.e. parasitaemia peak) dpi. The results

obtained with derivatives 16 and 24 are reflected in Figs 4 and 5, respectively.

As it can be seen in Fig. 4A, none of the treatments carried out with compound 16 suppressed the parasitaemia of acute infected

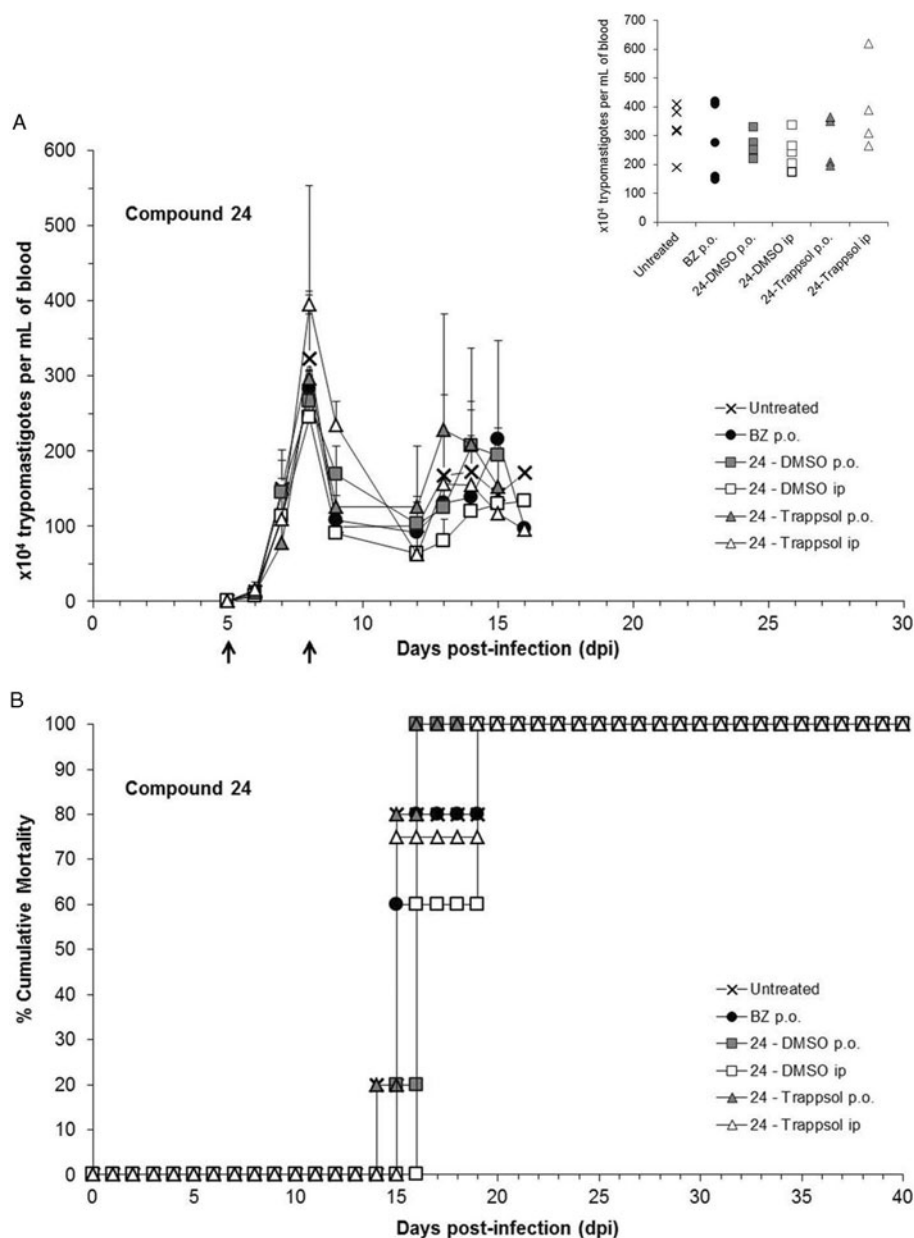


**Fig. 4.** Parasitaemia levels (A) and mortality rates (B) of different groups of male Swiss Webster mice ( $n=5$ ) treated with two unique doses of the 5-nitroindazole derivative 16, administered by using two different vehicles (i.e. 10% DMSO and 20% Trappsol) and given at 5th and 8th dpi (black arrows), either p.o. or ip. Results are expressed as the mean  $\pm$  s.d. Individual levels of parasitaemia at the peak day (i.e. 8th dpi) were included as an inset. \*Significant differences ( $P < 0.05$ ) with the untreated group, one-way ANOVA.

mice. Focusing on the results obtained at the day of the parasitaemia peak (i.e. 8th dpi), the combined therapy of this 5-nitroindazole derivative with the reference drug was the only effective regimen, reducing the parasitaemia levels more successfully (ca. 30%) than the administration of BZ alone (i.e. 12.48%). However, this combination did not protect animals against the mortality triggered by *T. cruzi* infection, which in this group happened between 15th and 19th dpi (Fig. 4B). Moreover, the ponderal curves registered for all the infected animals showed lower rates than that of the group of non-infected mice, pointing to an evident impact of the infection over the body weight (Supplementary Fig. S2). Generally, all the infected mice suffered important loss of body weight during the second week post-infection ( $P < 0.05$ ), being this fact undoubtedly connected with the beginning of mortality (Fig. 4B). However, the surviving mice – i.e. 20% ( $n=1/5$ ) of the animals belonging to the two groups orally treated – began to recover body weight during the fourth week post-infection, reaching at 28th dpi percentages of variation comparable to that of the non-infected group (Supplementary Fig. S2). Regarding the vehicles, the oral administration of derivative 16 led to similar profiles of effectiveness, mortality and variation of body weight,

independently of the approach (i.e. 10% DMSO or 20% Trappsol). Such a behaviour was also observed when this treatment was given ip (Fig. 4 and Supplementary Fig. S2). Concerning the effectiveness of compound 24, the best parasitaemia reduction at the peak day was obtained by the dilution of this derivative in 10% DMSO (v/v), which reached 17.80 and 24.23% of reduction when administered p.o. and ip, respectively. Otherwise, this latter compound showed no trypanocidal effect when 20% Trappsol (w/v) was selected as a vehicle, since all the treated mice (both, p.o. and ip) exhibited parasitaemia levels at 8th dpi similar to that of the untreated group (Fig. 5A). Besides, none of the schemes carried out with derivative 24 neither induced protection against the mortality associated to the parasitic infection (Fig. 5B) nor prevented the significant weight loss ( $P < 0.05$ ) ordinarily registered at 14th dpi for *T. cruzi*-infected animals (Supplementary Fig. S2).

These preliminary outcomes guided us to choose the most promising combination of each 5-nitroindazole derivative, vehicle and route, to be assayed in a subsequent experiment in which prolonged regimens of five consecutive doses of 25 mg kg<sup>-1</sup> administered at 5–9th dpi were studied. Accordingly, 10% DMSO (v/v)



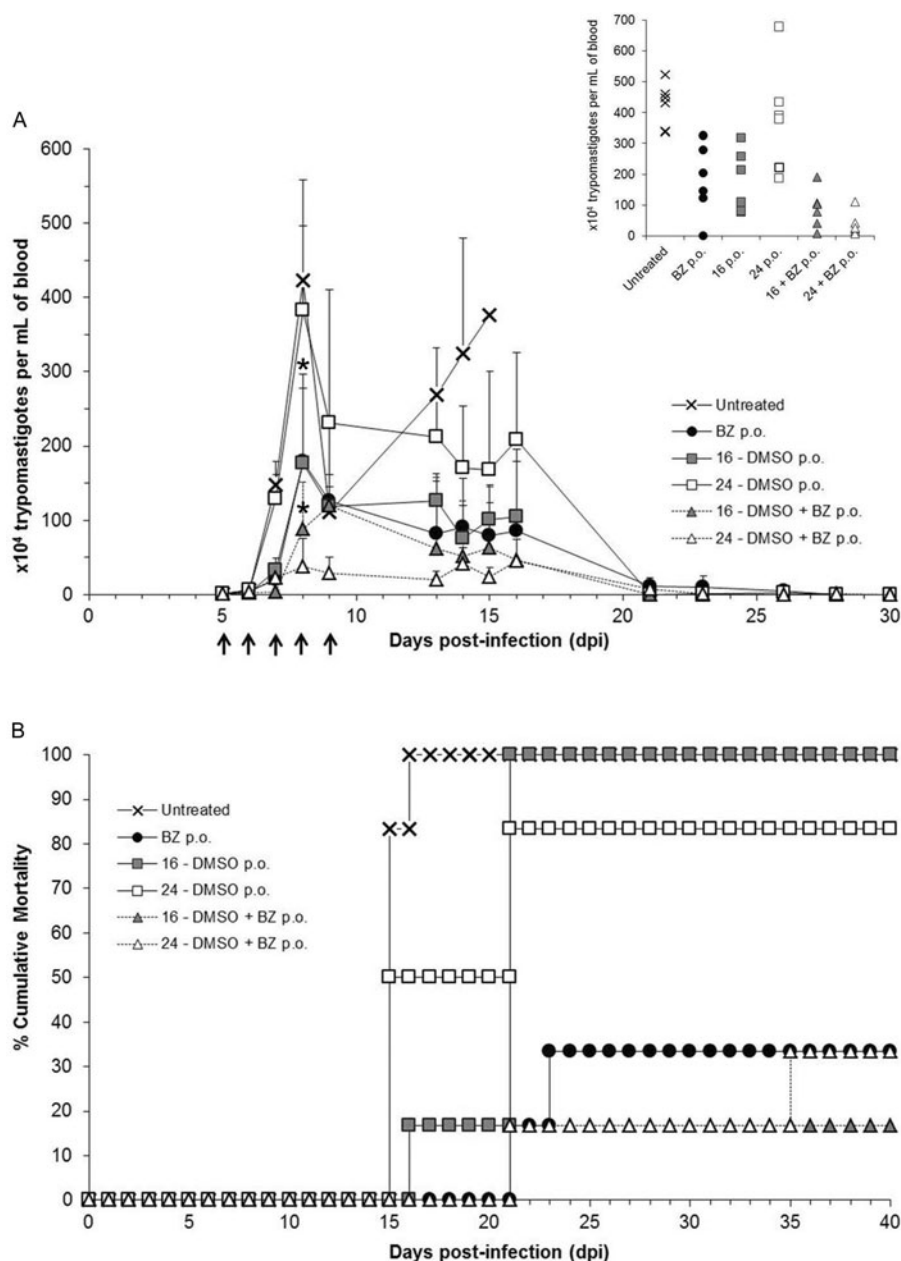
**Fig. 5.** Parasitaemia levels (A) and mortality rates (B) of different groups of male Swiss Webster mice ( $n=5$ ) treated with two unique doses of the 5-nitroindazole derivative 24, administered by using two different vehicles (i.e. 10% DMSO and 20% Trappsol) and given at 5th and 8th dpi (black arrows), either p.o. or ip. Results are expressed as the mean  $\pm$  s.d. Individual levels of parasitaemia at the peak day (i.e. 8th dpi) were included as an inset. \*Significant differences ( $P < 0.05$ ) with the untreated group, one-way ANOVA. Note:  $n=4$  for the group treated with 24 - Trappsol ip, since one of the animals initially included had no positive parasitaemia at 5th dpi and then, it was excluded from the experiment.

and the oral administration were selected as the most suitable vehicle and route, respectively (Figs 4 and 5). As reflected in Fig. 6A, none of these treatments was able to induce parasitaemia suppression in *T. cruzi*-infected mice. However, after giving the first three doses, derivative 16 attained ca. 60% of reduction in the parasitaemia levels of the peak day ( $P < 0.05$ ), being as effective as BZ. Otherwise, the effectiveness of derivative 24 did not improve with the application of this new regimen, since this treatment reached a minor parasitaemia reduction at 8th dpi (9.79%) and did not induce significant gains in the body weight of infected mice during the second week post-infection (Fig. 6A and Supplementary Fig. S3, respectively), unlike the reference drug ( $P < 0.05$ ). Regarding the mortality associated to the infection, these monotherapies did not reduce death rates, since the groups of mice treated with compounds 16 and 24 showed 0 ( $n=0/6$ ) and 16.67% ( $n=1/6$ ) of survival, respectively (Fig. 6B). However, both 5-nitroindazole derivatives exhibited a positive impact over *T. cruzi* infection when they were co-administered with the reference drug (Fig. 6A): the parasitaemia peak decreased 79.24% when compound 16 was given in combination with BZ ( $P < 0.05$ ) and 91.11% with the co-administration of derivative 24

and BZ ( $P < 0.05$ ). These two activity profiles were better than the one obtained with the administration of BZ alone, which reduced 57.61% the parasitaemia of the peak day. These combined treatments also protected mice against mortality, which did not occur until the third week post-infection (i.e. 21st dpi): in combination with BZ, derivatives 16 and 24 yielded 83.33 ( $n=5/6$ ) and 66.67% ( $n=4/6$ ) of survival, respectively (Fig. 6B). Moreover, both groups of mice that received these combined therapies did not suffer weight loss during the second week post-infection (Supplementary Fig. S3), showing at 14th dpi healthier profiles than the one typically exhibited by infected and untreated mice ( $P < 0.05$ ).

Finally, the cardiac function of the animals that entered into the chronic stage of the infection was explored by the study of ECG parameters and their comparison with those of the non-infected mice (Fig. 7). It is noteworthy to point out that at 120th dpi, the percentages of survival of those mice treated with BZ, either alone or in combination with derivatives 16 or 24, were the same previously obtained at 40th dpi (Fig. 6B), not occurring any deaths during the 40–120th dpi period. Indeed, these infected and treated animals did not exhibit significant





**Fig. 6.** Parasitaemia levels (A) and mortality rates (B) of different groups of male Swiss Webster mice ( $n=6$ ) treated with five consecutive doses (black arrows) of 5-nitroindazole derivatives 16 and 24, either in monotherapy or co-administered with BZ, vehiculated in 10% DMSO and given p.o. Results are expressed as the mean  $\pm$  s.d. Individual levels of parasitaemia at the peak day (i.e. 8th dpi) were included as an inset. \*Significant differences ( $P < 0.05$ ) with the untreated group, one-way ANOVA.

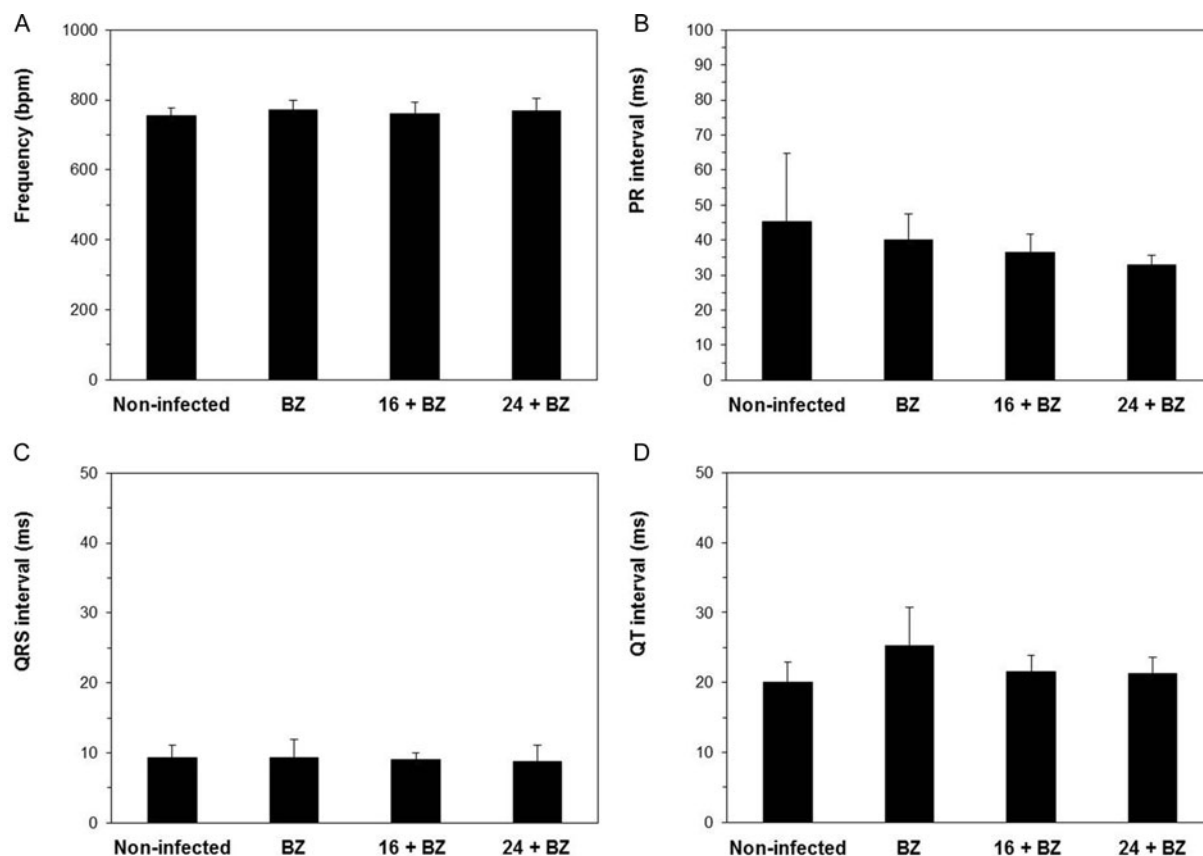
alterations in cardiac frequency, PR, QRS and QT intervals, when compared with the values shown by the group of non-infected mice (Fig. 7). Otherwise, these parameters could not be monitored for the group of infected and non-treated mice, since none of these animals overcame the acute experimental infection caused by *T. cruzi* (Fig. 6B).

## Discussion

*Trypanosoma cruzi* population is currently divided into six DTUs (TcI–TcVI), according to their genetic background, geographical location, ecotopes and mammalian hosts distributed along the different endemic areas (Zingales *et al.*, 2014). This high parasite diversity prompted researchers to investigate the association between the different *T. cruzi* strains and/or DTUs and drug susceptibility, since variable effectiveness of both reference antichagasic drugs occurs according to the geographical origin of patients (Coura and de Castro, 2002; Francisco *et al.*, 2017; Sales Junior *et al.*, 2017). In fact, DTU TcVI strains, such as CL Brener and Tulahuen, are representative drug-sensitive parasites, while those belonging to DTU TcI (e.g. Colombiana) are usually

designated as high drug-resistant strains. Among these naturally resistant parasites, *T. cruzi* Y (DTU TcII) has been defined as a moderately drug-resistant strain, according to the medium resistance it displays to both nifurtimox and BZ (Romanha *et al.*, 2010; Soeiro *et al.*, 2013). Recent studies conducted by Zingales *et al.* (2015) and Petravicius *et al.* (2019) have pointed to the involvement of specific ATP-binding cassette (ABC) transporters in the natural resistance of *T. cruzi* to BZ, according to the over-expression of one ABCG-like protein in BZ-resistant strains (e.g. Colombiana, DTU TcI), unlike in BZ-susceptible ones, such as CL Brener (DTU TcVI). These authors have also proposed variations in the levels of type-I nitroreductases (i.e. NTR-1 and Old Yellow Enzyme) among those mechanisms responsible for the different sensitivity of trypanosomatids to this reference drug (Petravicius *et al.*, 2019). However, a clear correlation between parasite DTUs and the presumable response of *T. cruzi* remains to be fully understood (Francisco *et al.*, 2017).

In previous works, our research group has introduced the 5-nitroindazole scaffold as a promising template for developing novel antichagasic compounds. Several 5-nitroindazole derivatives have demonstrated outstanding activity profiles, not only against



**Fig. 7.** Electrocardiographic parameters of the surviving mice obtained at 120th dpi:  $n = 6/6$  (non-infected mice),  $n = 4/6$  (BZ),  $n = 5/6$  (compound 16 plus BZ) and  $n = 4/6$  (compound 24 plus BZ). Frequency (A), PR (B), QRS (C) and QT (D) intervals. Bars represent the mean  $\pm$  s.e. \*Significant differences ( $P < 0.05$ ) with the group of non-infected mice, one-way ANOVA.

drug-sensitive *T. cruzi* strains (i.e. CL Brener and Tulahuén), but also towards the moderately drug-resistant Y strain, either *in vitro* (Fonseca-Berzal *et al.*, 2015, 2016a, 2018) or *in vivo* (Fonseca-Berzal *et al.*, 2014, 2017, 2019a, 2019b). Here, the effectiveness of two promising drug candidates, i.e. 5-nitroindazolinone derivatives 16 and 24, whose trypanocidal profiles *in vitro* against DTU TcVI parasites have been recently reported (Fonseca-Berzal *et al.*, 2016a, 2018), were deeper explored upon *T. cruzi* using the naturally resistant parasite strain Y (i.e. DTU TcII) under distinct experimental models, both *in vitro* and *in vivo*. Accordingly, we followed those recommendations that point to the importance of testing experimental compounds over DTUs TcII and TcVI parasites at the initial stage of the screening (Don and Ioset, 2014; Zingales *et al.*, 2014), providing this way new evidences of the potential that these 5-nitroindazolinone derivatives possess as putative trypanocidal agents.

In this framework, we have previously identified 16 and 24 as promising anti-*T. cruzi* compounds, according to the great activity these molecules showed against both epimastigotes ( $IC_{50} = 0.35$  and  $5.43 \mu M$ , respectively) and intracellular amastigotes ( $IC_{50} = 0.29$  and  $0.25 \mu M$ , respectively) of a DTU TcVI strain (Fonseca-Berzal *et al.*, 2016a, 2018). Interestingly, the trypanocidal profile that these derivatives displayed here over both replicative forms of this DTU TcII strain (Table 1) was quite similar, having no differences among the activity towards these two representative *T. cruzi* strains (i.e. CL Brener and Y). However, such an outstanding profile was not observed after treating *in vitro* the non-replicative form of *T. cruzi* (Table 1). This fact has also been detected with other molecules belonging to these two families of 5-nitroindazole derivatives (Fonseca-Berzal *et al.*, 2016a, 2018). This particular behaviour has not been observed in

5-nitroindazole derivatives that are substituted at the positions 1 and 3 of the heterocyclic ring, which show similar anti-*T. cruzi* profiles *in vitro* against the two clinically relevant forms of the parasite (Muro *et al.*, 2014; Martín-Escolano *et al.*, 2018), being this fact also appreciated in other 1,3-disubstituted 5-nitroindazoles previously studied by us (data not published). Otherwise, substitutions at the positions 1 and 3 of the 5-nitroindazole scaffold do not usually lead to such a remarkable  $IC_{50}$  over amastigotes and thus, these derivatives are generally not as active as compounds 16 and 24 against intracellular *T. cruzi* forms (Muro *et al.*, 2014; Martín-Escolano *et al.*, 2018).

After confirming *in vitro* the outstanding activity of these molecules towards intracellular forms of Y strain, both 16 and 24 were moved to a mouse model of acute infection caused by this DTU TcII strain, in order to explore their effectiveness *in vivo*. Prior to these assays, acute toxic effects manifested as a consequence of their administration to mice were successfully discarded (Fig. 3 and Supplementary Tables S1 and S2). In this regard, several studies of acute toxicity previously conducted by us, have explored the suitability of 10% DMSO (v/v) to solubilize increasing doses of 5-nitroindazolinones (Fonseca-Berzal *et al.*, 2017, 2019a, 2019b). However, some solubility problems were detected when this vehicle was selected for administering low water-soluble derivatives (Fonseca-Berzal *et al.*, 2017). In order to avoid these difficulties, 20% Trappsol (w/v) was chosen here, aiming to improve drug solubility and to obtain a proper administration of compounds 16 and 24. The results obtained in these experiments (Fig. 3 and Supplementary Tables S1 and S2) corroborated the lack of toxicity previously registered *in vitro* over both L929 (Fonseca-Berzal *et al.*, 2016a, 2018) and CC (Supplementary Fig. S1). In fact, none of the parameters recorded exhibited

significant alterations after concluding the total observation period of 48 h, pointing to healthy profiles for all the experimental groups (Supplementary Tables S1 and S2). Also, the cardiological monitoring of the animals and therefore, any change noticed in their ECG waves and intervals is particularly important in this kind of toxicological studies (Botelho et al., 2019). Certainly, such alterations were not recorded after the administration of these derivatives, either p.o. or ip (Fig. 3) and the animals showed ECG parameters similar to those previously found for healthy Swiss Webster mice (Campos et al., 2016). According to these results, which indicated NOAEL > 200 mg kg<sup>-1</sup> for both compounds – some solubility problems observed with compound 24 led to NOAEL > 100 mg kg<sup>-1</sup> when given ip (Supplementary Table S1), different protocols were applied administering to *T. cruzi*-infected mice non-toxic doses of 25 mg kg<sup>-1</sup>, as mono- and combined therapy with BZ. The reference drug was also given in suboptimal doses of 25 mg kg<sup>-1</sup>, which correspond to 1/4 of the regimen standardized for this model of acute infection: BZ optimal doses of 100 mg kg<sup>-1</sup> completely suppress the parasitaemia of *T. cruzi* Y-infected Swiss Webster mice, either with two unique administrations (de Araújo et al., 2014; Timm et al., 2014) or five consecutive daily doses (Fonseca-Berzal et al., 2014; Timm et al., 2014; da Silva et al., 2017; Simões-Silva et al., 2017, 2019). According to this, the administration of both derivatives and BZ in such a low dosage allows better comparison among the impact of these experimental treatments over *T. cruzi* infection (Martín-Escolano et al., 2018). Indeed, trypanocidal effectiveness was not so relevant when 16 and 24 were given alone in two unique doses of 25 mg kg<sup>-1</sup>, as happened with the reference drug (Figs 4 and 5). However, important effects were evidenced when these derivatives were co-administered with BZ in a prolonged scheme of five consecutive daily doses of 25 mg kg<sup>-1</sup> (Fig. 6). These combined treatments improved both the effectiveness and the survival rate observed when the respective compound (i.e. 16, 24 and also BZ) was given in monotherapy under the same regimen. Besides, CD commonly courses with important damages of the electrogenic cardiac system, being the ECG an important tool for the diagnosis and prognosis of the disease (Martinez et al., 2019). In fact, atrioventricular blockage and arrhythmias are frequent disturbances detected also in *T. cruzi*-infected mice (Campos et al., 2016). Here, those infected and treated animals that overcame the acute infection showed ECG tracings similar to those of the non-infected mice (Fig. 7), suggesting that these combined therapies of derivatives 16 and 24 with BZ protected mice against this kind of cardiac alterations. Considering all the results obtained *in vivo*, these monotherapy protocols conducted with both 5-nitroindazole derivatives did not lead to better effectiveness than BZ; however, the co-administration schemes showed superior anti-parasitic profile than the reference drug. Recently, other pre-clinical studies have revealed important findings of trypanocidal effectiveness *in vivo* by co-administering different compounds with the reference drug BZ (Guedes-da-Silva et al., 2019; Simões-Silva et al., 2019). The implementation of combined therapies is an alternative actually considered for the treatment of CD (Sales Junior et al., 2017; Ribeiro et al., 2020), and already in clinical use for combating many illnesses, including those triggered by parasitic infections, such as malaria (WHO, 2015) and sleeping sickness (WHO, 2019), among others.

### Concluding remarks

The outcomes introduced in the present work corroborate the 5-nitroindazole scaffold as a promising template for developing novel anti-*T. cruzi* drugs. Concretely, 1-(2-aminoethyl)-2-benzyl-5-nitro-1,2-dihydro-3*H*-indazol-3-one (hydrochloride) – compound

16 – and 1-(2-acetoxyethyl)-2-benzyl-5-nitro-1,2-dihydro-3*H*-indazol-3-one – compound 24 – bore out remarkable selectivity *in vitro* on the replicative forms of the moderately drug-resistant *T. cruzi* Y strain (DTU TcII). Furthermore, the lack of toxic effects that both derivatives induced *in vivo* and the effectiveness obtained with subcurative doses of 25 mg kg<sup>-1</sup>, encourage our research group to consider higher doses and/or more prolonged therapeutic regimens for further investigating the suitability of both 5-nitroindazolinone derivatives as alternative trypanocidal agents, administered either as monotherapy or in combination with reference antichagasic drugs. Regarding the latter, our findings also reinforce the plausible use of drug co-administration for treating CD, as already used in other parasitic diseases. Finally, other works directed to evaluate ADMETox properties of these molecules are desirable and planned, aiming to explore both the pharmacokinetics and safety profile of these 5-nitroindazole derivatives.

**Supplementary material.** The supplementary material for this article can be found at <https://doi.org/10.1017/S0031182020000955>.

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**Conflict of interest.** None.

**Ethical standards.** The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

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