Exploring the potential activity spectrum of two 5-nitroindazolinone prototypes on different *Trypanosoma cruzi* strains

CRISTINA FONSECA-BERZAL[1,2]*, PATRICIA BERNARDINO DA SILVA[3], CRISTIANE FRANÇA DA SILVA[3], MARIANE VASCONCELOS[3], MARCOS MEUSER BATISTA[3], JOSÉ A. ESCARIO[1,2], VICENTE J. ARÁN[1,4], ALICIA GÓMEZ-BARRIO[1,2] *and* MARIA DE NAZARÉ C. SOEIRO[3]

[1] *CEI Campus Moncloa, UCM-UPM & CSIC, Madrid, Spain*
[2] *Departamento de Parasitología, Facultad de Farmacia, Universidad Complutense de Madrid, Pza. Ramón y Cajal s/n, 28040 Madrid, Spain*
[3] *Laboratório de Biologia Celular, Instituto Oswaldo Cruz, Fiocruz, Av. Brasil 4365, 21040-900 Rio de Janeiro, Brazil*
[4] *Instituto de Química Médica (IQM), Consejo Superior de Investigaciones Científicas (CSIC), c/Juan de la Cierva 3, 28006 Madrid, Spain*

(*Received 3 July 2015; revised 7 August 2015; accepted 24 August 2015*)

SUMMARY

In the present study, the potential activity of two 5-nitroindazole derivatives previously proposed as suitable antichagasic prototypes was further evaluated on diverse *Trypanosoma cruzi* strains belonging to two discrete typing units (DTUs) frequently associated with human infection (i.e. DTUs TcII and TcVI). The trypanocidal profile that both 2-benzyl-1-propyl (22) and 2-benzyl-1-butyl (24) derivatives achieved on Tulahuen amastigotes (IC_{50} = 3·56 ± 0·99 and 6·31 ± 1·04 μM, respectively) correlates with that of formerly obtained on CL Brener, corroborating an outstanding activity on DTU TcVI parasites. Moreover, a sequential screening on extracellular and intracellular stages of *T. cruzi* Y (DTU TcII) demonstrated also the effectiveness of 22 and 24 over this strain on a similar range of activity (IC_{50} epimastigotes = 3·55 ± 0·47 and 7·92 ± 1·63 μM, IC_{50} amastigotes = 2·80 ± 0·46 and 9·02 ± 5·26 μM, respectively). These results, supported by a lack of toxicity registered over either L929 fibroblasts or primary cultures of cardiomyocytes, confirm that 5-nitroindazolinones 22 and 24 display great selectivity on both drug-sensitive (CL and Tulahuen) and drug-moderately resistant (Y) *T. cruzi* strains, and therefore, represent an important outcome in the research of Chagas disease chemotherapy.

Key words: Chagas disease, *Trypanosoma cruzi*, 5-nitroindazolinone, DTU, L929 fibroblasts, cardiomyocytes.

INTRODUCTION

Chagas disease (CD), caused by the kinetoplastid haemoflagellate *Trypanosoma cruzi*, is a parasitic infection naturally transmitted in 21 Latin American countries by the contaminated feces of blood-sucking bugs (i.e. triatomines). In the endemic area, CD provokes more than 7000 deaths per year and maintains over 25 million people at risk for the infection (WHO, 2015). Moreover, its emerging character in non-endemic areas due to population mobility and alternative routes of transmission (e.g. blood transfusion, organ donation, mother-to-child and through contaminated food) (Schmunis and Yadon, 2010) has contributed to the spreading of an illness that currently affects about 7 million people worldwide (WHO, 2015).

As one of the 17 tropical diseases defined by the WHO as neglected (WHO, 2015), strategies of research and development are focused on the finding of a suitable CD chemotherapy (Zingales *et al*. 2014). Many efforts have been made by institutions in order to develop new compounds potentially applicable to the treatment of CD, since the available chemotherapy relies on two old nitroheterocyclic drugs: benznidazole (BZ), the first-line treatment in most countries, and nifurtimox (NX) (WHO, 2015). Although these medicines are currently accepted to treat the acute and the early-chronic disease, both show limited effectiveness in long-term chronic infections (Urbina, 2015) and also exhibit undesirable side effects (Castro *et al*. 2006).

Concerning experimental chemotherapy research, in a previous study we proposed the 1,2-disubstituted 5-nitroindazolinone scaffold as prototype of antichagasic drug (Vega *et al*. 2012). Concretely, the 2-benzyl-1-propyl (22) and the 2-benzyl-1-butyl (24) derivatives (Fig. 1) achieved outstanding activity over the replicative stages of *T. cruzi* (i.e. epimastigotes and amastigotes) without toxicity on macrophages, what led to great selectivity on CL Brener strain (Vega *et al*. 2012; Fonseca-Berzal *et al*. 2014).

* Corresponding author: Departamento de Parasitología, Facultad de Farmacia, Universidad Complutense de Madrid, Pza. Ramón y Cajal s/n, Madrid 28040, Spain. E-mail: crfonseca@pdi.ucm.es

doi:10.1017/pao.2015.4

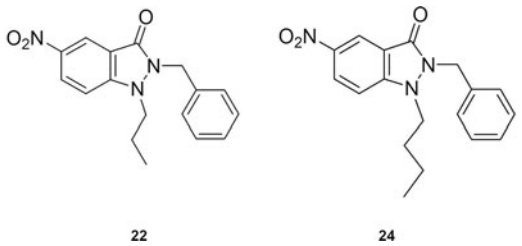

Fig. 1. Chemical structures of the prototypes 2-benzyl-5-nitro-1-propylindazolin-3-one (22) and 2-benzyl-1-butyl-5-nitroindazolin-3-one (24).

Trypanosoma cruzi populations have been classified into six discrete typing units (DTUs TcI–TcVI) according to molecular genetics, eco-epidemiological features and pathogenicity (Zingales *et al*. 2014). Moreover, susceptibility to reference drugs also varies among *T. cruzi* strains, with CL Brener and Tulahuen (TcVI) known as drug-sensitive, Y (TcII) as drug-moderately resistant and Colombian (TcI) as drug-resistant strain (Soeiro *et al*. 2013).

In this context, the Parasitology Department of UCM (Madrid, Spain), the Medicinal Chemistry Institute of CSIC (Madrid, Spain) and the Cellular Biology Laboratory of IOC/Fiocruz (Rio de Janeiro, Brazil) have worked in collaboration to further explore the trypanocidal spectrum of these candidates, following routinely screening procedures standardized by these laboratories. Consequently, the aims of the present study were: (i) to analyse the trypanocidal profile of prototypes 22 and 24 on other *T. cruzi* strains belonging to DTUs involved in human infection; (ii) to evaluate their activity in an *in vitro* golden model that uses primary cultures of cardiac cells as mammalian hosts since heart is one of the main targets for CD infection and inflammation; and (iii) to confirm their lack of toxicity over different mammalian cell cultures.

MATERIALS AND METHODS

Ethics

All procedures involving mice were carried out in accordance with the guidelines established by the Fiocruz Committee of Ethics for the Use of Animals (CEUA LW16/14).

Compounds

The synthesis of the two 5-nitroindazole derivatives assayed in the present work (Fig. 1) was previously described (Vega *et al*. 2012). The numbering of compounds used in this reference has been followed in the present paper. For all the *in vitro* assays, stock solutions of 22 and 24 were prepared in dimethyl sulfoxide and extemporaneously added to the cultures in a final concentration of the solvent non-toxic itself (<1%, v/v). BZ (Laboratório Farmacêutico do Estado de Pernambuco – LAFEPE, Brazil) was assayed as a reference drug.

Mammalian cell cultures

Primary cultures of embryonic cardiomyocytes (CM) were obtained from Swiss mice as previously described (Meirelles *et al*. 1986). After their purification, CM cultures were sustained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2·5 mM $CaCl_2$, 1 mM L-glutamine, 5% heat-inactivated fetal bovine serum (FBS) (30 min, 56 °C) and 2% chicken embryo extract. CM cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

Murine L929 fibroblasts were grown in plastic culture flasks (75 cm^2) using either Minimal Essential Medium (MEM) without phenol-red and supplemented as reported (Fonseca-Berzal *et al*. 2014) (MEMS) or RPMI-1640 without phenol-red supplemented with 10% heat-inactivated FBS and 2 mM glutamine (RPMIS). L929 cultures were maintained in a humidified 5% CO_2 atmosphere at 37 °C and subpassaged once a week. A 0·03% ethylenediaminetetraacetic acid (EDTA) and 0·05% trypsin in phosphate-buffered saline (PBS) solution was used for cell detachment.

Parasites

The Y strain of *T. cruzi*, originally isolated from an acute human case (Silva and Nussenzweig, 1953) and the Tulahuen strain, stably transfected with the *Escherichia coli* β-galactosidase gene *lacZ* (Buckner *et al*. 1996) were used throughout the experiments.

Regarding the Y strain, epimastigotes were maintained at 28 °C in supplemented liver infusion tryptose (LIT) medium as described in (Vega *et al*. 2012) and the axenic cultures continuously maintained in logarithmic growth by weekly passages. Bloodstream trypomastigotes (BT) of this strain were obtained by heart puncture from infected Swiss mice at the parasitaemia peak day and after their purification, resuspended in RPMI medium supplemented with 5% heat-inactivated FBS.

Concerning Tulahuen parasites, tissue culture-derived trypomastigotes (TCT) of this β-galactosidase-transfected strain were harvested in the supernatant of L929 cultures previously infected with invasive forms of *T. cruzi* and maintained in RPMIS at 37 °C in a humidified 5% CO_2 atmosphere (Romanha *et al*. 2010).

Epimastigote susceptibility assay

The activity on epimastigotes was evaluated by applying the resazurin assay previously standardized by Rolón *et al*. (2006). Log-phase epimastigotes in LIT medium were seeded in the culture tubes at

a density of 3×10^6 parasites mL^{-1} and maintained at 28 °C overnight to allow homogeneous growth. Afterwards, cultures were distributed in 96-well microplates (200 μL perwell) and incubated within the compounds for 48 h at 28 °C. Growth, medium and drug controls were also included in each assay and concentrations tested in triplicate. Finally, 20 μL of a resazurin solution in 1% PBS (3 mM, pH 7) was added per well and the plates maintained at 28 °C for another 5 h. Fluorescence intensity was measured with excitation (535 nm) and emission (590 nm) wavelengths (alamarBlue® Assay, U.S. Patent No. 5 501 959) and the results represent the percentage of epimastigote growth inhibition (% EGI). For each compound, the concentration that inhibits 50% of epimastigote growth (IC_{50}) was estimated by plotting the concentrations tested *vs* the % EGI. Selectivity indexes (SI) and potencies relative to BZ (RP) were also estimated.

Cytotoxicity assays

In order to detect any potential toxicity towards the host cell, cultures of CM and L929 were incubated within these compounds and the metabolic cell function was measured in the presence of resazurin-based indicators (PrestoBlue® and Resazurin sodium salt, respectively).

According to this, 100 μL of DMEM containing 6×10^4 CM per well were seeded in 96-well microplates previously coated with gelatin and incubated overnight at 37 °C in a humidified 5% CO_2 atmosphere. Afterwards, the medium was replaced by solutions of each compound in fresh DMEM and the plates were incubated either 24 or 48 h in the conditions aforementioned. Each concentration was evaluated in triplicate and controls of cellular growth were included in all the plates. Once the incubation concluded, both cell morphology and contractibility were examined by light microscopy and cellular viability evaluated by adding the redox indicator PrestoBlue® according to the manufacturer's instructions. After 5 h of incubation at 37 °C in a humidified 5% CO_2 atmosphere, the absorbance was read at 570 and 600 nm and the results were expressed as the percentage of cytotoxicity on CM ($\%C_{CM}$) (Romanha *et al*. 2010).

Moreover, the unspecific cytotoxicity over L929 was assayed in 96-well plates by seeding either 10×10^3 or 15×10^3 cells in 100 μL of MEM per well. In order to allow cell attachment, the plates were incubated for 3 h at 37 °C in a humidified 5% CO_2 atmosphere and then, the medium was replaced by 200 μL of compounds diluted in fresh MEM. Each concentration was tested in triplicate. Growth, medium and drug controls were also included in each plate. Fibroblasts were exposed to the compounds for 48, 72 and 96 h at 37 °C with 5% CO_2. Afterwards, 20 μL of a resazurin in 1% PBS solution (2 mM, pH 7) was added to each well and the plates were returned to the incubator for another 3 h. Finally, fluorescence intensity was read at 535 nm (excitation) and 590 nm (emission) and the results were expressed as $\%C_{L929}$ (Fonseca-Berzal *et al*. 2015).

For both assays, the concentration that inhibits 50% of cellular growth (LC_{50}) was estimated by plotting drug concentrations *vs* %C.

Intracellular amastigote susceptibility assays

The activity on intracellular amastigotes was evaluated by infecting either L929 fibroblasts with TCT (Tulahuen strain) or CM with BT (Y strain) in a 10:1 ratio (parasite:cell).

For the first bioassay, 100 μL of RPMIS containing 4000 L929 cells per well was seeded in 96-well tissue culture plates and maintained for 24 h at 37 °C in a humidified 5% CO_2 atmosphere. Afterwards, cells were incubated within TCT for another 2 h and then, non-penetrated parasites were discarded replacing the culture medium by fresh RPMIS. In order to establish the infection, the plates were maintained during 48 h at 37 °C and 5% CO_2 and then, the medium was replaced by solutions of each compound in fresh RPMIS. Each concentration was evaluated in triplicate. Controls of infection and cell growth were also included and the plates were incubated for 96 h at the same conditions of temperature and humidity. After this period, 50 μL of 500 μM chlorophenol red glycoside in 0·5% Nonidet P40 was added to each well and the plates were incubated for 18 h at 37 °C. Finally, the absorbance was read at 570 nm and the results were expressed as the percentage of amastigote growth inhibition (%AGI) (Romanha *et al*. 2010).

To assay these compounds over Y strain amastigotes, 100 000 CM per well were seeded in 24-well tissue culture plates provided with round coverslips previously coated with gelatin and then, maintained in DMEM overnight at 37 °C in a humidified 5% CO_2 atmosphere. After 24 h of parasite–host cell interaction, the infected cultures were washed to remove non-internalized trypomastigotes and then, incubated for another 48 h within the compounds diluted in fresh DMEM. Next, the cultures were fixed with Bouin's fixative and stained with Giemsa. The mean number of infected CM and the mean number of parasites per infected CM were scored in 400 host cells by duplicate. Only parasites with characteristic nuclei and kinetoplast were counted, since the irregular ones were considered as parasites undergoing death. Finally, activity results were estimated by calculating the inhibition of the endocytic index (EI) (da Silva *et al*. 2007).

In each assay, compounds IC_{50} and IC_{90} (concentration that inhibits 50 and 90% of amastigote proliferation or EI, respectively) were estimated by plotting concentrations *vs* %AGI or %EI. SI over the respective host cell and RP were also calculated.

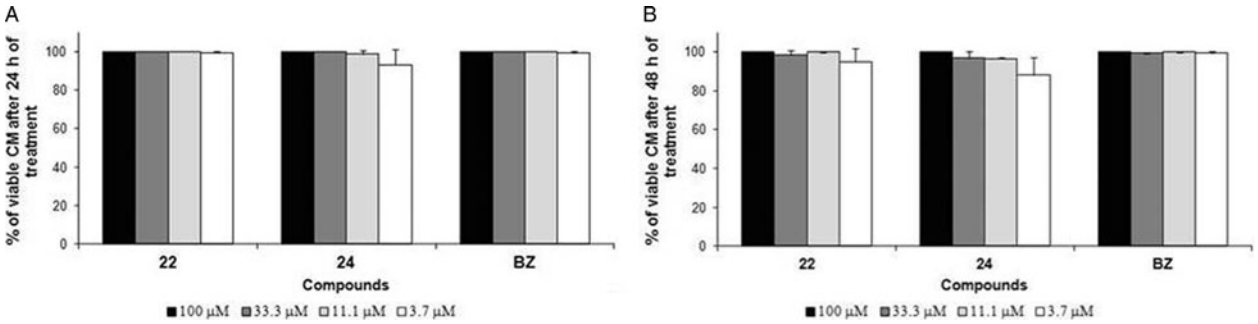

Fig. 2. Toxic effect of the 5-nitroindazolinones over primary cultures of CM after 24 h (A) and 48 h (B) of treatment.

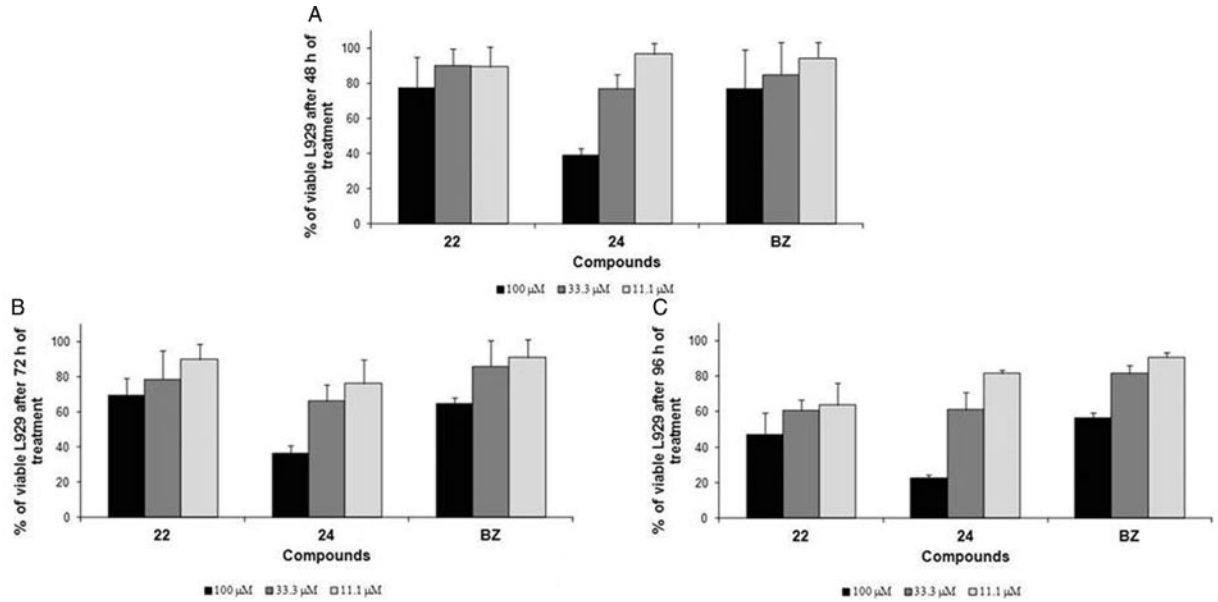

Fig. 3. Toxic effect of the 5-nitroindazolinones over L929 cells after 48 h (A), 72 h (B) and 96 h (C) of treatment.

All the *in vitro* assays were run in the same conditions at least twice separately. For each assay, the results are expressed as the mean value of activity ± standard deviation (S.D.).

Statistical analysis

SPSS Statistics software (version 20, IBM) was used for the statistical analysis. The non-parametric Kruskal–Wallis test was applied to compare compounds activity between the different strains, as well as with that of BZ. $P < 0{\cdot}05$ was considered statistically significant.

RESULTS

Toxicity on mammalian host cells

Since both CM and L929 were used as *T. cruzi* host cell, the potential toxicity of 22 and 24 over these mammalian cells was assessed *in vitro*. As Fig. 2 reflects, no toxicity was perceived on the treated CM, and therefore, percentages of cell viability greater than 85% were registered in all the cases. In fact, both derivatives achieved LC_{50} values higher than 100 μM after the incubation times assayed (i.e. 24 and 48 h). Moreover, the light microscopy analysis revealed neither alteration in cell morphology nor affectation in contractibility as a treatment consequence.

Nevertheless, both compounds achieved a different cytotoxic profile on L929 fibroblasts. After 48 h of drug–cell contact, concentrations higher than 33·3 μM led to a pronounced loss of cellular viability that varied in a time-dependent manner. Likewise, BZ induced higher toxicity on this mammalian cell line, showing a response similar to that of derivative 22 (Fig. 3 and Table 1). However, at the highest concentration tested and after completing the three incubation times assayed on L929, no differences were detected between the toxic profile of 5-nitroindazolinones and BZ ($P > 0{\cdot}05$).

Activity on epimastigotes (Y strain)

As a primary screening, the inhibitory effect of derivatives 22 and 24 was evaluated on axenic cultures of epimastigotes. Both compounds displayed a better trypanocidal profile compared with that of

Table 1. Toxic effect of derivatives 22 and 24 on L929 cells

	48 h		72 h		96 h	
Compound	%C[a]	LC_{50} (μM)[b]	%C	LC_{50} (μM)	%C	LC_{50} (μM)
22	22·74 ± 17·74	>100	30·70 ± 9·98	>100	52·66 ± 12·34	86·57 ± 10·77
24	60·97 ± 3·94	79·79 ± 4·64	63·59 ± 4·26	67·44 ± 5·48	77·39 ± 1·75	55·34 ± 7·89
BZ	35·13 ± 14·33	>100	35·17 ± 3·27	>100	43·52 ± 2·79	>100

* Significant differences ($P < 0{\cdot}05$) compared with BZ (Kruskal–Wallis test).
[a] Results expressed as the mean value of cytotoxicity (%C) at the highest concentration tested (100 μM) ± standard deviation (S.D.) of three independent experiments ($n = 3$).
[b] The concentration causing 50% of cellular lethality (LC_{50}) was estimated by plotting drug concentrations *vs* %C. The results are expressed as the mean ± S.D. of three independent experiments ($n = 3$).

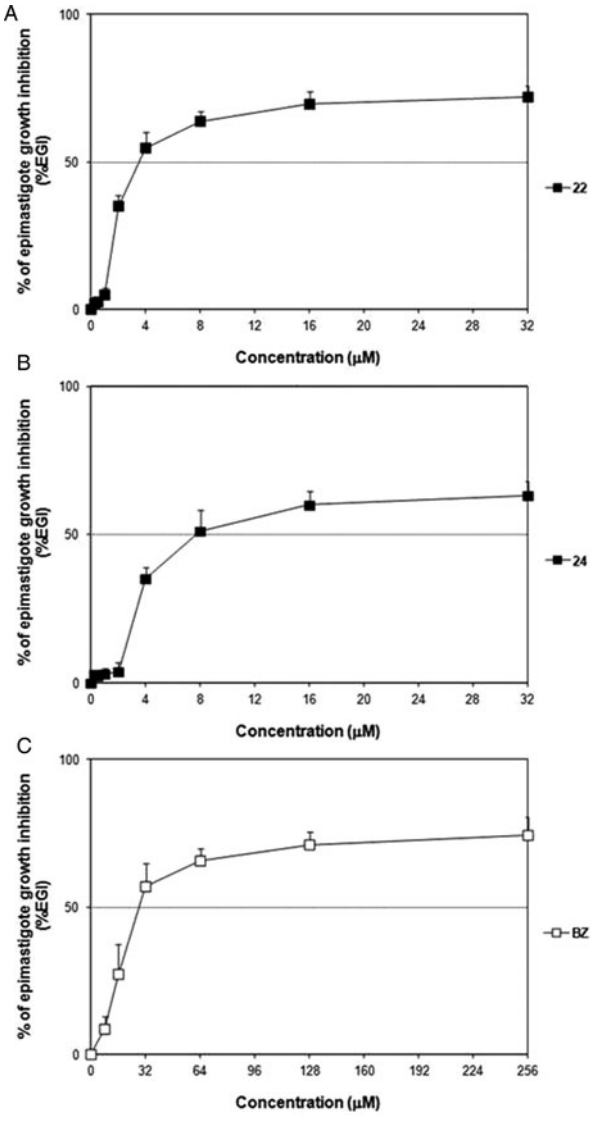

Fig. 4. Dose–response curves of 22 (A), 24 (B) and BZ (C) on epimastigotes (Y strain).

BZ (Fig. 4). They obtained improved IC_{50} values and remarkable SI on the extracellular parasite (Table 2), significantly better in the case of derivative 22 ($P < 0{\cdot}05$). Otherwise, the potency of these molecules could not be determined at this level, since their IC_{90} were reached at concentrations superior than the highest one assayed.

Activity on intracellular amastigotes

The inhibitory effect of 22 and 24 was further evaluated over Tulahuen and Y strain amastigotes grown in L929 and CM cultures, respectively. According to the results of cytotoxicity (Table 1), concentrations higher than 50 μM were not tested at this level. Although both compounds displayed slightly lower dose–response curves upon Tulahuen parasites compared with that of BZ (Fig. 5 and Table 3), they reached outstanding SI on the intracellular stage and were as potent ($P > 0{\cdot}05$) as the reference drug (Table 3).

Regarding the activity upon Y strain amastigotes, although compound 22 achieved similar IC_{50} values for both the strains ($P > 0{\cdot}05$), it accomplished a better IC_{90} after a 2-day treatment of Y-infected cells and a nearly suppression of the EI at 25 μM (%EI = 99·43 ± 0·32%) (Fig. 6), showing a trypanocidal profile similar to that of BZ (Table 4). Otherwise, derivative 24 was not only less effective but also with lower potency on this latter strain (Table 4). However, at 25 μM it obtained a considerable reduction in both the percentage of infected cells and the mean number of amastigotes per infected cell, as the inhibition in the EI reflects (%EI ca. 71%) (Figs 6 and 7).

DISCUSSION

The selection of diverse *T. cruzi* strains and clones for *in vitro* drug screening is an important point to consider in the early research of putative candidates for CD chemotherapy (Zingales *et al*. 2014). In fact, the different effectiveness that BZ and NX display among divergent strains is one of the aspects on which the current Chagas therapy failure resides (Urbina, 2015). Although a clear correlation between *T. cruzi* genetic variability and its presumable response to drugs has not been evidenced (Moraes *et al*. 2014), strategies for drug screening

Table 2. Trypanocidal activity of 22 and 24 on Y strain epimastigotes expressed as IC_{50}, IC_{90} and SI

Compound	IC_{50} (μM)[a]	IC_{90} (μM)	LC_{50} CM (μM)[b]	SI[c]
22	3·55 ± 0·47*,**	>32	>100	>28·17
24	7·92 ± 1·63**	>32	>100	>12·62
BZ	28·00 ± 4·64	>256	>100	>3·57

* Significant differences ($P < 0·05$) compared with BZ (Kruskal–Wallis test).
** Significant differences ($P < 0·05$) compared with the activity on CL-B5 epimastigotes (see Vega *et al*. 2012) (Kruskal–Wallis test).
[a] The concentration causing 50 and 90% of EGI (IC_{50} and IC_{90}, respectively) was estimated by plotting drug concentrations *vs* %EGI. The results are expressed as the mean ± S.D. of three independent experiments ($n = 3$).
[b] After 48 h of drug treatment.
[c] Selectivity indexes are defined as SI = LC_{50} (48 h CM cells)/IC_{50} Y epimastigotes.

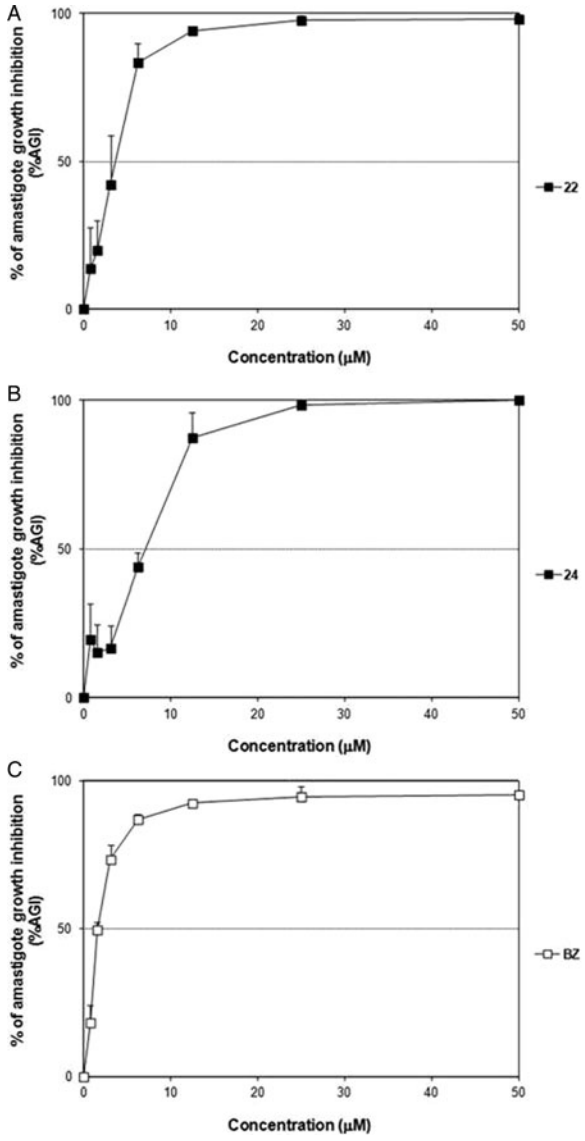

Fig. 5. Dose–response curves of 22 (A), 24 (B) and BZ (C) on intracellular amastigotes (Tulahuen strain) grown in L929 cultures.

should focus its performance on parasite DTUs more often associated with human infection (DTUs TcI, TcII, TcV and TcVI) (Zingales *et al*. 2014).

In previous studies, indazole-based compounds have demonstrated great effectiveness on different *T. cruzi* strains (reviewed in Aguilera-Venegas *et al*. 2013). Concretely, several series of 5-nitroindazole derivatives have achieved important outcomes exhibiting notable anti-*T. cruzi* activity (Arán *et al*. 2005; Boiani *et al*. 2009; Vega *et al*. 2012; Muro *et al*. 2014), being some of these molecules proposed to trigger reactive oxygen species (ROS)-based mechanisms of action (Folch-Cano *et al*. 2010).

In this framework, we have formerly identified the 5-nitroindazolinones 22 and 24 as promising antichagasic candidates, since the great activity they achieved on epimastigotes ($IC_{50} = 0·93$ and 1·17 μM, respectively) and intracellular amastigotes ($IC_{50} < 1$ and 3·71 μM, respectively) of *T. cruzi* CL Brener (Vega *et al*. 2012; Fonseca-Berzal *et al*. 2014). The results of this primary screening on a β-galactosidase-transfected clone (Buckner *et al*. 1996) with a biological behaviour similar to that of the parental CL strain (TcVI) (Le-Senne *et al*. 2002), prompted us to perform a deeper study of their broad activity on other *T. cruzi* strains (Zingales *et al*. 2014). Consequently, further screening procedures were carried out on intracellular forms of Tulahuen strain to confirm their activity on TcVI parasites, as well as over both extracellular and intracellular stages of *T. cruzi* Y, representative strain of TcII.

Moreover, an analysis of compounds relative cytotoxicity was simultaneously conducted. Although both candidates did not exert any toxic effect on J774 macrophages after 24 h of drug exposure (Vega *et al*. 2012), it is important to verify this fact over those mammalian cells hosting the parasite in subsequent trypanocidal assays (Fonseca-Berzal *et al*. 2014). In addition, either CM or fibroblasts are preferred as host cell for such an experimental model, since both are *T. cruzi* target cells more strictly involved in the pathology of the disease and only some parasite isolates concentrate infection in the mononuclear phagocytic system (Teixeira *et al*. 2006). Moreover, heart is an important target for infection and inflammation in CD pathology and

Table 3. Trypanocidal activity of 22 and 24 on intracellular amastigotes of Tulahuen strain expressed as IC_{50}, IC_{90} and SI. Compounds RP were also estimated

Compound	IC_{50} (μM)[a]	IC_{90} (μM)	LC_{50} L929 (μM)[b]	SI[c]	RP[d]
22	3·56 ± 0·99	9·60 ± 1·17	86·57 ± 10·77	24·32	1·00
24	6·31 ± 1·04*	15·55 ± 4·86	55·34 ± 7·89	8·77	0·62
BZ	1·66 ± 0·10	9·63 ± 0·37	>100	>60·24	–

* Significant differences ($P < 0·05$) compared with BZ (Kruskal–Wallis test).
** Significant differences ($P < 0·05$) compared with the activity on CL-B5 amastigotes (see Fonseca-Berzal *et al*. 2014) (Kruskal–Wallis test).
[a] The concentration causing 50 and 90% of amastigote growth inhibition (IC_{50} and IC_{90}, respectively) was estimated by plotting drug concentrations *vs* %AGI. The results are expressed as the mean ± S.D. of three independent experiments ($n = 3$).
[b] After 96 h of drug treatment (see Table 1).
[c] Selectivity indexes are defined as SI = LC_{50} (96 h L929 cells)/IC_{50} Tulahuen amastigotes.
[d] Relative potencies are defined as RP = IC_{90} BZ/IC_{90} tested compound.

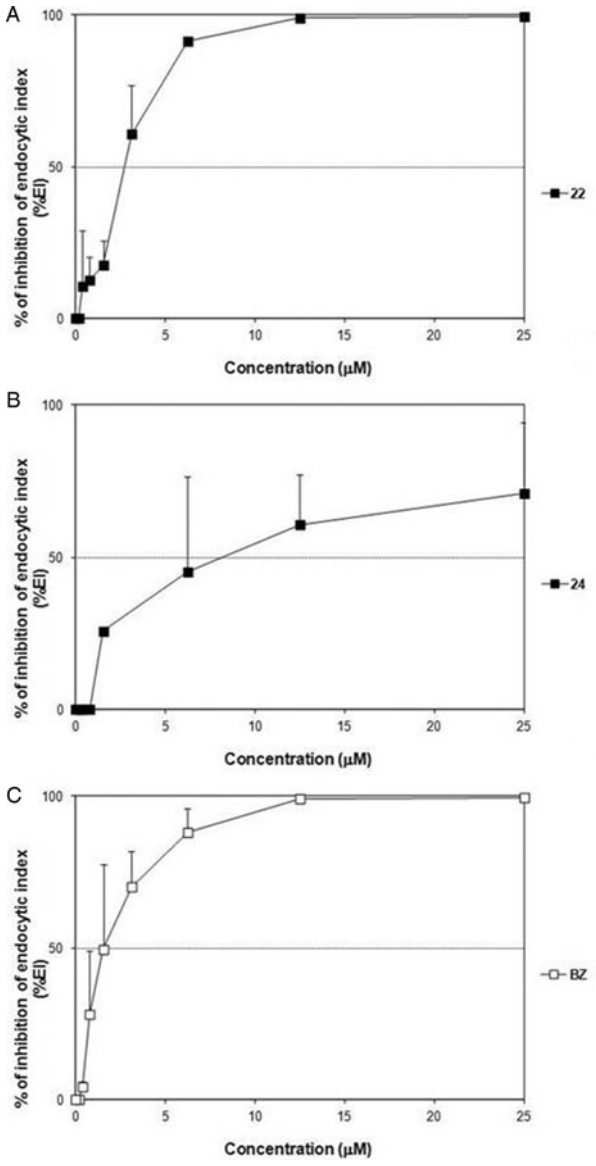

Fig. 6. Dose–response curves of 22 (A), 24 (B) and BZ (C) on intracellular amastigotes (Y strain) grown in CM cultures.

then, pre-clinical studies using these cells are desirable to exclude potential drug candidates with cardiotoxicity characteristics (da Silva *et al*. 2007). According to this, we tested compounds 22 and 24 over uninfected L929 fibroblasts and primary cultures of CM in order to rule out the toxic effects. Neither compound 22 nor 24 resulted in toxicity on CM after completing 48 h of incubation (Fig. 2). Otherwise, high concentrations of both derivatives seemed to induce a loss of L929 viability that increased in a time-dependent manner, showing a behaviour similar to that of BZ ($P > 0·05$) (Fig. 3 and Table 1). It could happen as a result of the different nature of both mammalian cell cultures (i.e. primary cultures and continuous cell line, respectively) (Duran-Rehbein *et al*. 2014), having obtained a similar pattern with the reference drug (Figs 2 and 3). In fact, differences related to susceptibility and sensitivity using the same drug have been also reported according to the mammalian cell type and the respective viability assay employed (Zwolak, 2014; Emter and Natsch, 2015; Xu *et al*. 2015). Herein, the noticed differences in drug sensitivity detected among fibroblasts and cardiac cells may arise due to the different cytotoxic assays performed (Resazurin sodium salt × PrestoBlue®) and from the dissimilar impact susceptibility of each target cell (fibroblasts *vs* cardiac cells).

Several studies have reported no apparent DTU association regarding epimastigote susceptibility to both reference drugs (Boiani *et al*. 2006; Moreno *et al*. 2010; Zingales *et al*. 2014) agreeing this statement with the results we achieved with BZ on CL-B5 ($IC_{50} = 27·32\ \mu$M) (Vega *et al*. 2012) and Y strain ($IC_{50} = 28·00\ \mu$M) ($P > 0·05$). The variation in glutathione content (free or mostly conjugated as trypanothione) found among *T. cruzi* strains has been also proposed as an explanation of their different susceptibility to both NX and BZ (Repetto *et al*. 1996), since these reduced thiols play an important role in the free radical detoxification mechanisms of trypanosomatids (Irigoin *et al*. 2008). Moreover, no relevant differences in glutathione content were formerly established among Tulahuen, CL and Y epimastigotes (Moncada *et al*. 1989) presuming a comparable response of these strains to BZ, what

Table 4. Trypanocidal activity of 22 and 24 on intracellular amastigotes of Y strain expressed as IC_{50}, IC_{90} and SI. Compounds RP were also estimated

Compound	IC_{50} (μM)[a]	IC_{90} (μM)	LC_{50} CM (μM)[b]	SI[c]	RP[d]
22	2·80 ± 0·46	6·02 ± 0·30	>100	>35·71	1·07
24	9·02 ± 5·26	>25	>100	>11·08	<0·25
BZ	1·77 ± 1·11	6·46 ± 1·34	>100	>56·50	–

* Significant differences ($P < 0·05$) compared with BZ (Kruskal–Wallis test).
** Significant differences ($P < 0·05$) compared with the activity on CL-B5 (see Fonseca-Berzal *et al*. 2014) and Tulahuen amastigotes (Kruskal–Wallis test).
[a] The concentration causing 50 and 90% of EI inhibition (IC_{50} and IC_{90}, respectively) was estimated by plotting drug concentrations *vs* %EI. The results are expressed as the mean ±S.D. of two independent experiments ($n = 2$).
[b] After 48 h of drug treatment.
[c] Selectivity indexes are defined as SI = LC_{50} (48 h CM cells)/IC_{50} Y amastigotes.
[d] Relative potencies are defined as RP = IC_{90} BZ/IC_{90} tested compound.

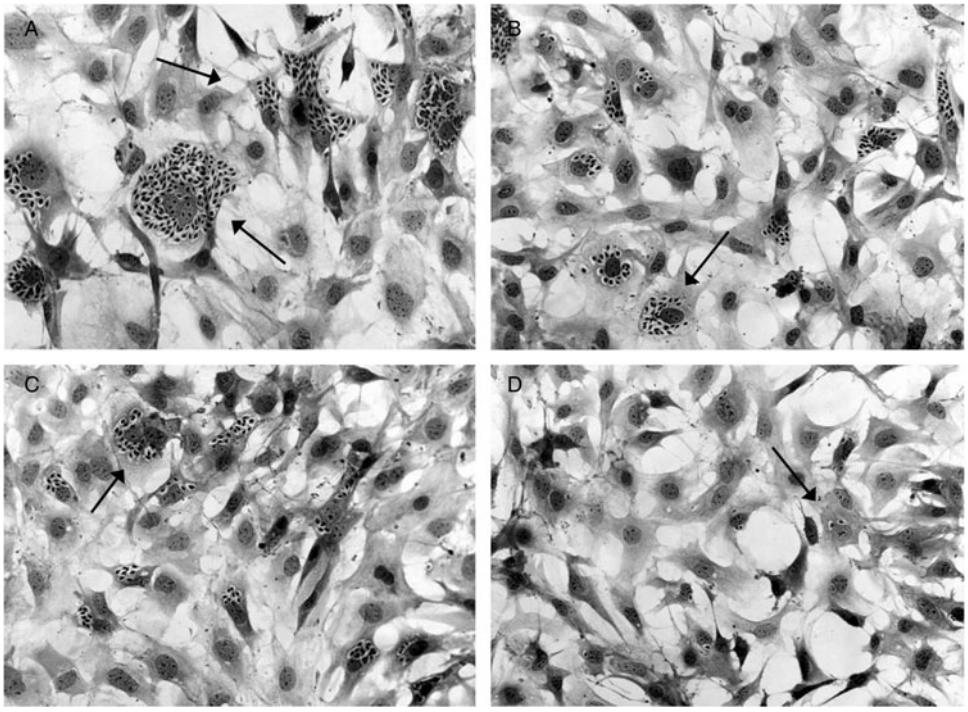

Fig. 7. Light microscopy analysis of CM infected with Y strain and treated for 48 h with 6·25 μM (B), 12·50 μM (C) and 25 μM (D) of derivative 24, as well as untreated cells (A). The black arrows indicate intracellular amastigotes.

correlates with the similar profiles herein obtained (Table 2). Although the epimastigote is not the clinically relevant form of *T. cruzi*, the easy maintenance this model offers (Muelas *et al*. 2001) and the identification of an intracellular epimastigote-like form as intermediate stage within the mammalian host (Faucher *et al*. 1995), support the performance of pre-screening models on this stage of *T. cruzi*. Additionally, a correlation between both the activity on epimastigotes and amastigotes occurs (Vega *et al*. 2012; Fonseca-Berzal *et al*. 2014). However, compounds IC_{50} on these both stages usually differ in several orders of magnitude (Moreno *et al*. 2010). Undoubtedly, posterior inhibition assays on infective forms (i.e. trypomastigotes and amastigotes) must be carried out *in vitro* before advancing compounds to *in vivo* assays (Romanha *et al*. 2010), since different drug susceptibility can be registered among different stages from the same *T. cruzi* stock (Moraes *et al*. 2014).

According to these facts, we evaluated the effectiveness of derivatives 22 and 24 upon the three main forms of *T. cruzi* Y strain, which is routinely used in our laboratories for *in vitro* and *in vivo* drug screening models (Castillo-Garit, *et al*. 2012; da Silva *et al*. 2012; Araújo *et al*. 2014; Fonseca-Berzal *et al*. 2015). The trypanocidal profile both prototypes accomplished on this strain was quite similar to that of over CL-B5 parasites (Vega *et al*. 2012; Fonseca-Berzal *et al*. 2014). However, our compounds displayed lower activity towards epimastigotes of Y strain ($P < 0·05$), unlike BZ (Table 2). This fact may be connected with the moderately

resistance to nitroderivative drugs attributed to *T. cruzi* Y (Romanha *et al*. 2010), contrasting with the susceptible CL Brener (Le-Senne *et al*. 2002). Moreover, 22 and 24 seemed to be more selective than BZ on extracellular epimastigotes (SI_{22} > 28·17, SI_{24} > 12·62 and SI_{BZ} > 3·57), since they got better effectiveness on this stage too (Table 2).

Following the criteria proposed by DNDi (Don and Ioset, 2014), compounds that obtain IC_{50} values inferior than 10 μM on intracellular amastigotes of TcVI or TcII are identified as putative *hits* for CD chemotherapy, likewise our two derivatives. These compounds formerly displayed such an outstanding activity on CL-B5 (Fonseca-Berzal *et al*. 2014) now confirmed in the phenotypic assay on Tulahuen ($P > 0{\cdot}05$) (Table 3), both TcVI strains. Besides, derivatives 22 and 24 also fulfilled it over Y amastigotes (TcII) grown in cardiac cells *in vitro* (Table 4). In fact, both compounds proved to be 10-fold more active on the Y strain than toxic to mammalian cells (Don and Ioset, 2014), successfully overpassing this preliminary *hit stage* (SI_{22} > 35·71 and SI_{24} > 11·08).

Nevertheless, both nitroheterocycles not only displayed great activity on *T. cruzi* models, but also were considered as suitable templates for the design of novel anti-*Trypanosoma brucei* agents (Arán *et al*. 2012). However, the activity they achieved against blood trypomastigotes of both African (Arán *et al*. 2012) and American trypanosomes (data not shown) was lower than the obtained on the multiplying stages of *T. cruzi*. In the present study, our compounds achieved an improved trypanocidal profile on intracellular TcII parasites, likewise BZ (Tables 2 and 4). The activity displayed by the reference drug on *T. cruzi* Y, could be again related to variations in thiol concentration among different stages of a unique strain (Maya *et al*. 1997). According to this, the higher the content of reduced thiols (i.e. epimastigotes > trypomastigotes > amastigotes), the less susceptible to BZ (IC_{50} value on epimastigotes > trypomastigotes > amastigotes). Actually, in a previous *in vivo* study we found that 22 and 24 did not entirely suppress parasitaemia through the acute disease in mice, but obtained a considerably reduction in BT levels after concluding a 5-day treatment (Fonseca-Berzal *et al*. 2014). The activity on BT was higher for derivative 24 either *in vivo* (Fonseca-Berzal *et al*. 2014) or *in vitro* models (data not shown), which conversely was the less active derivative on intracellular *T. cruzi*.

The results compiled in the present study corroborate these two 5-nitroindazolinone derivatives as putative antichagasic prototypes. Both compounds bore out remarkable effectiveness on the clinically relevant stage of TcII and TcVI strains with a lack of toxicity on diverse host cells that resulted in great selectivity on *T. cruzi*. Concretely, the 2-benzyl-5-nitro-1-propylindazolin-3-one (22) showed trypanocidal and cytotoxic profiles similar to those of the reference drug BZ. Further investigation directed to explore the mechanism of action triggered by these compounds is currently underway.

ACKNOWLEDGEMENTS

The authors thank the Programme for Technological Development in Tools for Health (PDTIS-Fiocruz) for the facilities. C.F.-B., J.A.E., V.J.A and A.G.-B. acknowledge M.N.C.S for kindly accepting C.F.-B. predoctoral stay in LBC/IOC/Fiocruz.

FINANCIAL SUPPORT

The present study was supported by grants from Fundação Carlos Chagas Filho de Amparo a Pesquisa do Estado do Rio de Janeiro (FAPERJ), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação Oswaldo Cruz, PDTIS, PROEP/CNPq/Fiocruz, CAPES. M.N.C.S. is research fellows of CNPq and CNE researchers. This work was also supported by the UCM-BSCH Research Group 'Terapia Antiparasitaria' (ref. no. 911120). Research by C.F.-B. was supported by a PICATA predoctoral fellowship and a PICATA predoctoral mobility grant of the Moncloa Campus of International Excellence (UCM-UPM & CSIC).

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.

REFERENCES

Aguilera-Venegas, B., Olea-Azar, C., Arán, V. J. and Speisky, H. (2013). Indazoles: a new top seed structure in the search of efficient drugs against *Trypanosoma cruzi*. *Future Medicinal Chemistry* **5**, 1843–1859.

Arán, V. J., Ochoa, C., Boiani, L., Buccino, P., Cerecetto, H., Gerpe, A., González, M., Montero, D., Nogal, J. J., Gómez-Barrio, A., Azqueta, A., López de Ceráin, A., Piro, O. E. and Castellano, E. E. (2005). Synthesis and biological properties of new 5-nitroindazole derivatives. *Bioorganic and Medicinal Chemistry* **13**, 3197–3207.

Arán, V. J., Kaiser, M. and Dardonville, C. (2012). Discovery of nitroheterocycles active against African trypanosomes. *In vitro* screening and preliminary SAR studies. *Bioorganic and Medicinal Chemistry Letters* **22**, 4506–4516.

Araújo, J. S., da Silva, C. F., Batista, D. G., da Silva, P. B., Batista, M. M., Aiub, C. A. F., da Silva, M. F., Araújo-Lima, C. F., Banerjee, M., Farahat, A. A., Stephens, C. E., Kumar, A., Boykin, D. W. and Soeiro, M. N. C. (2014). *In vitro* and *in vivo* studies of the biological activity of novel arylimidamides against *Trypanosoma cruzi*. *Antimicrobial Agents and Chemotherapy* **58**, 4191–4195.

Boiani, M., Boiani, L., Denicola, A., Torres de Ortiz, S., Serna, E., Vera de Bilbao, N., Sanabria, L., Yaluff, G., Nakayama, H., Rojas de Arias, A., Vega, C., Rolón, M., Gómez-Barrio, A., Cerecetto, H. and González, M. (2006). 2*H*-Benzimidazole 1,3-dioxide derivatives: a new family of water-soluble anti-trypanosomatid agents. *Journal of Medicinal Chemistry* **49**, 3215–3224.

Boiani, L., Gerpe, A., Arán, V. J., Torres de Ortiz, S., Serna, E., Vera de Bilbao, N., Sanabria, L., Yaluff, G., Nakayama, H., Rojas de

Arias, A., Maya, J. D., Morello, J. A., Cerecetto, H. and González, M. (2009). *In vitro* and *in vivo* antitrypanosomatid activity of 5-nitroindazoles. *European Journal of Medicinal Chemistry* **44**, 1034–1040.

Buckner, F. S., Verlinde, C. L. M. J., La Flamme, A. C. and Van Voorhis, W. C. (1996). Efficient technique for screening drugs for activity against *Trypanosoma cruzi* using parasites expressing β-galactosidase. *Antimicrobial Agents and Chemotherapy* **40**, 2592–2597.

Castillo-Garit, J. A., del Toro-Cortés, O., Kouznetsov, V. V., Puentes, C. O., Romero Bohórquez, A. R., Vega, M. C., Rolón, M., Escario, J. A., Gómez-Barrio, A., Marrero-Ponce, Y., Torrens, F. and Abad, C. (2012). Identification in silico and *in vitro* of novel trypanosomicidal drug-like compounds. *Chemical Biology and Drug Design* **80**, 38–45.

Castro, J. A., Montalto de Mecca, M. and Bartel, L. C. (2006). Toxic side effects of drugs used to treat Chagas' disease (American trypanosomiasis). *Human and Experimental Toxicology* **25**, 471–479.

da Silva, C. F., Batista, D. G., Oliveira, G. M., de Souza, E. M., Hammer, E. R., da Silva, P. B., Daliry, A., Araújo, J. S., Britto, C., Rodrigues, A. C., Liu, Z., Farahat, A. A., Kumar, A., Boykin, D. W. and Soeiro, M. N. C. (2012). *In vitro* and *in vivo* investigation of the efficacy of acrylimidamide DB1831 and its mesylated salt form -DB1965- against *Trypanosoma cruzi* infection. *PLoS ONE* **7**, e30356.

da Silva, C. F., Batista, M. M., Mota, R. A., de Souza, E. M., Stephens, C. E., Som, P., Boykin, D. W. and Soeiro, M. N. C. (2007). Activity of 'reversed' diamidines against *Trypanosoma cruzi* '*in vitro*'. *Biochemical Pharmacology* **73**, 1939–1946.

Don, R. and Ioset, J. R. (2014). Screening strategies to identify new chemical diversity for drug development to treat kinetoplastid infections. *Parasitology* **141**, 140–146.

Duran-Rehbein, G. A., Vargas-Zambrano, J. C., Cuéllar, A., Puerta, C. J. and González, J. M. (2014). Mammalian cellular culture models of *Trypanosoma cruzi* infection: a review of the published literature. *Parasite* **21**, 38.

Emter, R. and Natsch, A. (2015). A fast Resazurin-based live viability assay is equivalent to the MTT-test in the KeratinoSens assay. *Toxicology In Vvitro* **29**, 688–693.

Faucher, J. F., Baltz, T. and Petry, K. G. (1995). Detection of an 'epimastigote-like' intracellular stage of *Trypanosoma cruzi*. *Parasitology Research* **81**, 441–443.

Folch-Cano, C., Olea-Azar, C., Arán, V. J. and Díaz-Urrutia, C. (2010). ESR and electrochemical study of 1,2-disubstituted 5-nitroindazolin-3-ones and 2-substituted 3-alkoxy-5-nitro-2*H*-indazoles: reactivity and free radical production capacity in the presence of biological systems. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy* **75**, 375–380.

Fonseca-Berzal, C., Escario, J. A., Arán, V. J. and Gómez-Barrio, A. (2014). Further insights into biological evaluation of new anti-*Trypanosoma cruzi* 5-nitroindazoles. *Parasitology Research* **113**, 1049–1056.

Fonseca-Berzal, C., Palmeiro-Roldán, R., Escario, J. A., Torrado, S., Arán, V. J., Torrado-Santiago, S. and Gómez-Barrio, A. (2015). Novel solid dispersions of benznidazole: preparation, dissolution profile and biological evaluation as alternative antichagasic drug delivery system. *Experimental Parasitology* **149**, 84–91.

Irigoin, F., Cibils, L., Comini, M. A., Wilkinson, S. R., Flohe, L. and Radi, R. (2008). Insights into the redox biology of *Trypanosoma cruzi*: Trypanothione metabolism and oxidant detoxification. *Free Radical Biology and Medicine* **45**, 733–742.

Le-Senne, A., Muelas-Serrano, S., Fernández-Portillo, C., Escario, J. A. and Gómez-Barrio, A. (2002). Biological characterization of a β-galactosidase expressing clone of *Trypanosoma cruzi* CL strain. *Memórias do Instituto Oswaldo Cruz* **97**, 1101–1105.

Maya, J. D., Repetto, Y., Agosin, M., Ojeda, J. M., Téllez, R., Gaule, C. and Morello, A. (1997). Effects of nifurtimox and benznidazole upon glutathione and trypanothione content in epimastigote, trypomastigote and amastigote forms of *Trypanosoma cruzi*. *Molecular and Biochemical Parasitology* **86**, 101–106.

Meirelles, M. N., de Araújo-Jorge, T. C., Miranda, C. F., de Souza, W. and Barbosa, H. S. (1986). Interaction of *Trypanosoma cruzi* with heart muscle cells: ultrastructural and cytochemical analysis of endocytic vacuole formation and effect upon myogenesis *in vitro*. *European Journal of Cell Biology* **41**, 198–206.

Moncada, C., Repetto, Y., Aldunate, J., Letelier, M. E. and Morello, A. (1989). Role of glutathione in the susceptibility of *Trypanosoma cruzi* to drugs. *Comparative Biochemistry and Physiology. Part C: Comparative Pharmacology* **94**, 87–91.

Moraes, C. B., Giardini, M. A., Kim, H., Franco, C. H., Araujo-Junior, A. M., Schenkman, S., Chatelain, E. and Freitas-Junior, L. H. (2014). Nitroheterocyclic compounds are more efficacious than CYP51 inhibitors against *Trypanosoma cruzi*: implications for Chagas disease drug discovery and development. *Scientific Reports* **4**, 4703.

Moreno, M., D'ávila, D. A., Silva, M. N., Galvão, L. M., Macedo, A. M., Chiari, E., Gontijo, E. D. and Zingales, B. (2010). *Trypanosoma cruzi* benznidazole susceptibility *in vitro* does not predict the therapeutic outcome of human Chagas disease. *Memórias do Instituto Oswaldo Cruz* **105**, 918–924.

Muelas, S., Di Maio, R., Cerecetto, H., Seoane, G., Ochoa, C., Escario, J. A. and Gómez-Barrio, A. (2001). New thiadiazine derivatives with activity against *Trypanosoma cruzi* amastigotes. *Folia Parasitologica* **48**, 105–108.

Muro, B., Reviriego, F., Navarro, P., Marín, C., Ramírez-Macías, I., Rosales, M. J., Sánchez-Moreno, M. and Arán, V. J. (2014). New perspectives on the synthesis and antichagasic activity of 3-alkoxy-1-alkyl-5-nitroindazoles. *European Journal of Medicinal Chemistry* **74**, 124–134.

Repetto, Y., Opazo, E., Maya, J. D., Agosin, M. and Morello, A. (1996). Glutathione and trypanothione in several strains of *Trypanosoma cruzi*: effect of drugs. *Comparative Biochemistry and Physiology. Part B: Biochemistry and Molecular Biology* **115**, 281–285.

Rolón, M., Vega, C., Escario, J. A. and Gómez-Barrio, A. (2006). Development of resazurin microtiter assay for drug sensibility testing of *Trypanosoma cruzi* epimastigotes. *Parasitology Research* **99**, 103–107.

Romanha, A. J., Castro, S. L., Soeiro, M. N. C., Lannes-Vieira, J., Ribeiro, I., Talvani, A., Bourdin, B., Blum, B., Olivieri, B., Zani, C., Spadafora, C., Chiari, E., Chatelain, E., Chaves, G., Calzada, J. E., Bustamante, J. M., Freitas-Junior, L. H., Romero, L. I., Bahia, M. T., Lotrowska, M., Soares, M., Andrade, S. G., Armstrong, T., Degrave, W. and Andrade, Z. A. (2010). *In vitro* and *in vivo* experimental models for drug screening and development for Chagas disease. *Memórias do Instituto Oswaldo Cruz* **105**, 233–238.

Schmunis, G. A. and Yadon, Z. E. (2010). Chagas disease: a Latin American health problem becoming a world health problem. *Acta Tropica* **115**, 14–21.

Silva, L. H. and Nussenzweig, V. (1953). Sobre uma cepa de *Trypanosoma cruzi* altamente virulenta para o camundongo branco. *Folha Clinica e Biologica* **20**, 191–207.

Soeiro, M. N. C., de Souza, E. M., da Silva, C. F., Batista, D. G., Batista, M. M., Pavão, B. P., Araújo, J. S., Aiub, C. A. F., da Silva, P. B., Lionel, J., Britto, C., Kim, K., Sulikowski, G. and Hargrove, T. Y. (2013). *In vitro* and *in vivo* studies of the antiparasitic activity of sterol 14α-demethylase (CYP51) inhibitor VNI against drug-resistant strains of *Trypanosoma cruzi*. *Antimicrobial Agents and Chemotherapy* **57**, 4151–4163.

Teixeira, A. R., Nascimento, R. J. and Sturm, N. R. (2006). Evolution and pathology in Chagas disease – a review. *Memórias do Instituto Oswaldo Cruz* **101**, 463–491.

Urbina, J. A. (2015). Recent clinical trials for the etiological treatment of chronic Chagas disease: advances, challenges and perspectives. *Journal of Eukaryotic Microbiology* **62**, 149–156.

Vega, M. C., Rolón, M., Montero-Torres, A., Fonseca-Berzal, C., Escario, J. A., Gómez-Barrio, A., Gálvez, J., Marrero-Ponce, Y. and Arán, V. J. (2012). Synthesis, biological evaluation and chemometric analysis of indazole derivatives. 1,2-disubstituted 5-nitroindazolinones, new prototypes of antichagasic drug. *European Journal of Medicinal Chemistry* **58**, 214–227.

World Health Organization (2015). *Investing to Overcome the Global Impact of Neglected Tropical Diseases*, Third WHO Report on Neglected Tropical Diseases. Department of Control of Neglected Tropical Diseases, World Health Organization, Geneva.

Xu, M., McCanna, D. J. and Sivak, J. G. (2015). Use of the viability reagent PrestoBlue in comparison with alamarBlue and MTT to assess the viability of human corneal epithelial cells. *Journal of Pharmacological and Toxicological Methods* **71**, 1–7.

Zingales, B., Miles, M. A., Moraes, C. B., Luquetti, A., Guhl, F., Schijman, A. G. and Ribeiro, I. (2014). Drug discovery for Chagas disease should consider *Trypanosoma cruzi strain* diversity. *Memórias do Instituto Oswaldo Cruz* **109**, 828–833.

Zwolak, I. (2014). Comparison of three different cell viability assays for evaluation of vanadyl sulphate cytotoxicity in a Chinese hamster ovary K1 cell line. *Toxicology and Industrial Health*. doi:10.1177/0748233714544190.