

Repurposing strategies for Chagas disease therapy: the effect of imatinib and derivatives against *Trypanosoma cruzi*

Research Article

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Abstract

Chagas disease (CD) is a neglected parasitic condition endemic in the Americas caused by *Trypanosoma cruzi*. Patients present an acute phase that may or not be symptomatic, followed by lifelong chronic stage, mostly indeterminate, or with cardiac and/or digestive progressive lesions. Benznidazole (BZ) and nifurtimox are the only drugs approved for treatment but not effective in the late chronic phase and many strains of the parasite are naturally resistant. New alternative therapy is required to address this serious public health issue. Repositioning and combination represent faster, and cheaper trial strategies encouraged for neglected diseases. The effect of imatinib (IMB), a tyrosine kinase inhibitor designed for use in neoplasias, was assessed *in vitro* on *T. cruzi* and mammalian host cells. In comparison with BZ, IMB was moderately active against different strains and forms of the parasite. The combination IMB + BZ in fixed-ratio proportions was additive. Novel 14 derivatives of IMB were screened and a 3,2-difluoro-2-phenylacetamide (3e) was as potent as BZ on *T. cruzi* but had low selectivity index. The results demonstrate the importance of phenotypic assays, encourage the improvement of IMB derivatives to reach selectivity and testify to the use of repurposing and combination in drug screening for CD.

Introduction

Chagas disease (CD) is a neglected parasitic infection that affects more than six million people worldwide, most of them living in the Americas in extreme poverty without access to diagnosis or proper treatment (WHO, 2015; Chatelain, 2016). Caused by the protozoan *Trypanosoma cruzi*, CD is a silent and progressive disease with two phases: acute and chronic (Chatelain, 2016). During the acute phase, the patient may present mild flu-like symptoms or not, and after 6–9 weeks, enters the long-lasting chronic phase that is predominantly indeterminate (70% of the diagnosed cases), opposing to 30–40% of the patients, that can, years or decades later, develop clinic signs characterized by cardiac and/or digestive pathology (Prata, 2001; Rassi *et al.*, 2010). The therapy for CD is based in two drugs, benznidazole (BZ) and nifurtimox, both in use for more than five decades. However, both present severe side-effects, limited efficacy on the later chronic phase besides the occurrence of naturally resistant parasite strains to these nitroderivatives (Bermudez *et al.*, 2016).

Nowadays, drug repurposing strategies have been used in attempt to find alternative therapies for diseases that lack aetiological or adequate drug treatment (Nwaka and Hudson, 2006). The approach is especially relevant for the neglected diseases reducing time and budget in drug discovery process (Ashburn and Thor, 2004). In addition to that, combined therapy is as well an interesting strategy for enabling the simultaneous action towards more than one target, to overcome natural or acquired resistance (Ashburn and Thor, 2004; Sun *et al.*, 2016; Cha *et al.*, 2018).

Imatinib (IMB) is a rationally designed BCR-ABL tyrosine kinase (TK) inhibitor broadly used for neoplasias, such as chronic myeloid leukaemia and gastrointestinal stromal tumour (Rix *et al.*, 2007; Cruz-Rico *et al.*, 2013; Musumeci *et al.*, 2015). New analogues and derivatives of IMB have been synthesized and screened not only for neoplasias, but also for parasitic pathologies like malaria (Pathak *et al.*, 2015), schistosomiasis (Beckmann *et al.*, 2014; Buro *et al.*, 2014), filariasis (O’Connell *et al.*, 2015), leishmaniasis (Wetzel *et al.*, 2012), human African trypanosomiasis (Behera *et al.*, 2014) and CD (Engel *et al.*, 2010).

The scope of our study was to explore drug repositioning by evaluating the potential trypanocidal activity of IMB, also assessing its use in combination with BZ. The phenotypic activity of 14 novel IMB derivatives against the different forms and strains of *T. cruzi* was also investigated.

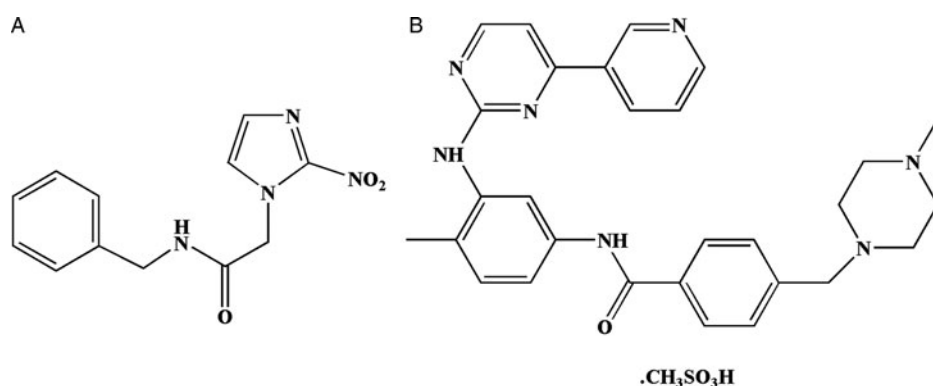


Fig. 1. Chemical structures of benznidazole (A) and imatinib mesylate (B).

Materials and methods

Drugs

BZ (Fig. 1) was purchased from Laboratório Farmacêutico do Estado de Pernambuco (Recife, Pernambuco, Brazil). IMB mesylate (Fig. 1) and the derivatives (1a–c, 1e, 2a–e and 3a–e – Fig. 2) were provided by Dr Núbia Boechat from the Laboratório de Síntese Orgânica – Instituto de Tecnologia em Fármacos/Fundação Oswaldo Cruz (Farmanguinhos/Fiocruz) (Rio de Janeiro, Rio de Janeiro, Brazil) and synthesized as reported (Azevedo *et al.*, 2017). Stock solutions in dimethyl sulfoxide were prepared w/v with the final concentration never exceeding 0.6%, which does not exert mammalian host cell toxicity as reported (Timm *et al.*, 2014). The highest concentration was 50 μM as some of the derivative molecules slightly precipitated (light microscopy observation) using higher concentrations.

Mammalian cell cultures

Mouse fibroblasts from L929 cell line (4×10^3 cell well⁻¹ into 96-well plates) were cultivated in RPMI-1640 medium (pH 7.2–7.4) without phenol red (Gibco BRL) supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine at 37 °C (Romanha *et al.*, 2010). Primary cardiac cell cultures (cc) were prepared from hearts isolated from 18–20 days old mice embryos and seeded in 96- and 24-well plates previously coated with 0.01% gelatin (Meirelles *et al.*, 1986).

Parasites

Trypomastigotes of Tulahuen strain (DTU VI) expressing the β -galactosidase gene from *Escherichia coli* were obtained from the supernatant of infected L929 cell line cultures, as described (Buckner *et al.*, 1996; Romanha *et al.*, 2010). Bloodstream trypomastigotes (BT) of Y strain (DTU II) were obtained at the parasitaemia peak from infected Swiss Webster mice and then resuspended in Dulbecco modified Eagle medium (DMEM), supplemented with 10% FBS (Meirelles *et al.*, 1986; Batista *et al.*, 2010). Culture-derived trypomastigotes (CT) of the Y strain were obtained from the supernatant of L929 cc (10:1 parasite: cell ratio) as described (Batista *et al.*, 2010).

Cell toxicity assessment

Non-infected cc and L929 cultures were incubated at 37 °C for 24–48 and 96 h, respectively, with increasing concentrations of each compound (up to 50 μM) diluted in RPMI. The morphology, and the spontaneous contractibility of cc was evaluated by light microscopy and cellular viability was determined by the PrestoBlue (cc) and AlamarBlue (L929) tests, as standardized (Romanha *et al.*, 2010; Simões-Silva *et al.*, 2016). The results

were expressed as the difference in reduction between treated and non-treated cells according to the manufacturer's instructions, and the value of LC₅₀ (minimum concentration that reduces the cellular viability by 50%) was determined. Also, toxicity on mammalian cells was assessed using combination schemes of IMB with BZ as described below (item 2.5.2). Selectivity index was expressed as the ratio between the mean values of the CC₅₀ for host cells, and the EC₅₀ for the parasites or infected cc (SI: CC₅₀/EC₅₀). All SIs were done comparing always the same time of drug exposure for each parasites and mammalian cells: 24 h for BT and CT, 48 h for intracellular and extracellular amastigote forms of Y strain, and 96 h for intracellular forms of Tulahuen strain, as reported (Batista *et al.*, 2010; Romanha *et al.*, 2010).

Trypanocidal activity

Monotherapy

BT and CT of Y strain (5×10^6 mL⁻¹) were incubated for 2 and 24 h at 37 °C in RPMI with 1:3 serial dilutions of the compounds (0–50 μM) for determination of parasite death rates, through the direct quantification of live parasites by light microscopy. The EC₅₀ (compound concentration that reduces the number of parasites by 50%) was then calculated (Timm *et al.*, 2014; Simões-Silva *et al.*, 2016). For the analyses of compound effect on intracellular forms, *T. cruzi*-infected L929 cultures (Tulahuen strain) were incubated for 96 h at 37 °C with each compound using 1:3 serial dilutions (0–50 μM) in RPMI. After the incubation, 500 μM chlorophenol red- β -D-galactopyranoside in 0.5% Nonidet P40 was added to each well (final concentration 50 μM) and the plate incubated for 18 h at 37 °C. The absorbance was measured at 570 and 600 nm using a spectrophotometer. Controls with uninfected cells and infected cells, both treated only with vehicle and/or with BZ, were run in parallel for each plate in order to determine the EC₅₀ values (Romanha *et al.*, 2010). To test the potency against other parasite strain, the selected compounds were evaluated on the infection of cc using the Y strain. After 24 h of interaction (10:1 parasite and host cell ratio), the cc were incubated for 48 h using 1:3 serial dilutions of the compounds (0–50 μM), rinsed with saline, fixed with Bouin, stained with Giemsa and examined by light microscopy (Simões-Silva *et al.*, 2016). The percentage of infected host cells was determined, as well as the number of parasites per cell and the infection index, which represents the product of the multiplication between the percentage of infection and the number of parasites per cell. Then, the EC₅₀ values were determined based on the infection indexes.

Regarding the effect on extracellular amastigotes (Y strain), the parasites were purified from the supernatant of highly infected cardiac cc (parasite: host cell ratio: 50:1) (De Souza *et al.*, 2010). Then, 5×10^6 parasites mL⁻¹ were incubated for 48 h at 37 °C with increasing concentrations of the studied compounds

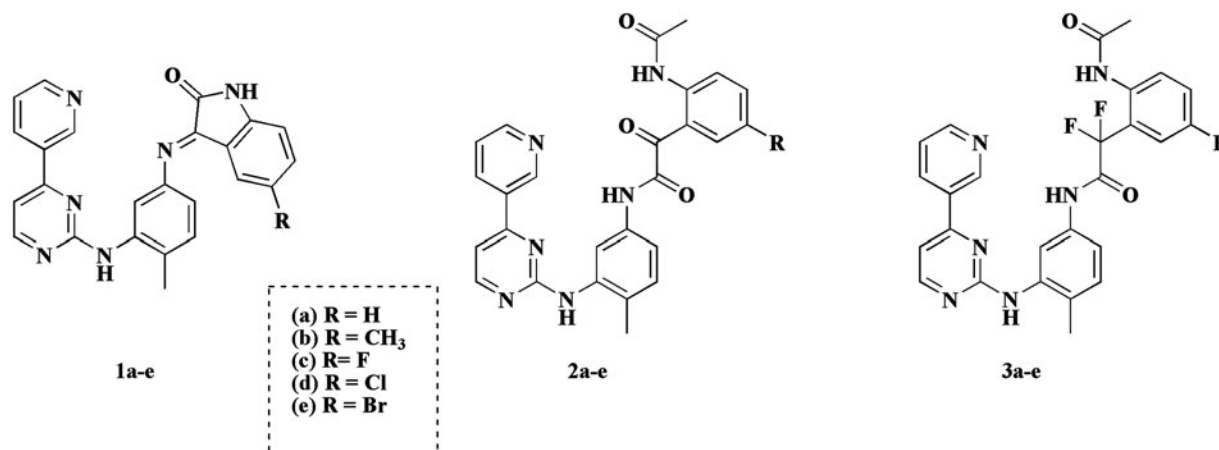


Fig. 2. Chemical structures of imatinib derivatives.

($\leq 50 \mu\text{M}$) and parasite death rates quantified by light microscopy for EC_{50} determination (De Souza et al., 2010). The maximum time of drug exposure was set according to the characteristics of parasite viability [for trypomastigotes the maximum was 24 h, since longer *in vitro* cultivation decreases viability (BT) or induces cell differentiation (CT)], and due to the previous standardized protocols for each parasite strain (Batista et al., 2010; Romanha et al., 2010).

Combined therapy

Drug interactions were investigated using a fixed-ratio method (Fivelman et al., 2004) by combining IMB with BZ, in L929 cc infected with Tulahuen strain expressing β -galactosidase, according to the same protocol above described. Predetermined EC_{50} values were used to determine the top concentrations of the individual ratios ensuring that it fell in the midpoint of a seven-point 2-fold dilution series. The fixed ratios of 5:0, 4:1, 3:2, 2:3, 1:4 and 0:5 were used, as reported (Simões-Silva et al., 2016).

Determination of FIC index, isobologram preparation and nature of drug interaction

Fractional inhibitory concentrations (FICs) and the sum of FICs ($\sum\text{FICs}$) were calculated as follows: $\text{FIC of IMB} = \text{EC}_{50} \text{ of IMB in combination} / \text{EC}_{50} \text{ of IMB in monotherapy}$. The same equation was applied to the partner drug BZ. The $\sum\text{FICs} = \text{FIC}_{\text{IMB}} + \text{FIC}_{\text{BZ}}$. An overall $\sum\text{FICs}$ was determined and used to classify the nature of each interaction. Isobolograms were built by plotting the EC_{50} of IMB against the EC_{50} of BZ (Diniz et al., 2013; Simões-Silva et al., 2016). $\sum\text{FICs} \leq 0.5 = \text{synergism}$; $0.5 < \sum\text{FICs} \leq 4.0 = \text{additive (no interaction)}$; $\sum\text{FICs} > 4.0 = \text{antagonism}$.

Statistical analysis

The assays were done in at least duplicate and the results were on average from three independent experiments. Analysis of variance tests were performed and the significance level set for $P \leq 0.05$ only for those compounds that presented higher potency than the reference drugs.

Results

Following our well-established flowchart, the activity against intracellular forms of *T. cruzi* (Tulahuen strain – DTU VI) was done using L929-infected cc treated or not with IMB and BZ in serial dilutions (0–50 μM) and readout using colorimetric analysis. As presented in Table 1, IMB displayed a moderate trypanocidal

Table 1. *In vitro* trypanocidal activity ($\text{EC}_{50} - \mu\text{M}$) of benzimidazole and imatinib against amastigote forms (Tulahuen and Y strains of *Trypanosoma cruzi*, intra and extracellular sources), as well as cytotoxicity ($\text{CC}_{50} - \mu\text{M}$) on different mammalian cells and their corresponding selectivity indexes (SI)

	Mean \pm s.d.	
	Benzimidazole	Imatinib
<i>T. cruzi</i> amastigote forms (strains and sources) and mammalian cells		
CC_{50} L929	>50	38.3 ± 0.2
EC_{50} intracellular (Tulahuen)	4.1 ± 1.3	24.8 ± 7.4
SI ^a	>12	1.5
CC_{50} cardiac cells	>50	>50
EC_{50} intracellular (Y)	2.8 ± 1.9^b	20.0 ± 4.4
SI ^c	>18	>2.5
CC_{50} cardiac cells	>50	>50
EC_{50} extracellular ^d (Y)	nd	30.0 ± 4.4
SI ^b	nd	>1.7

nd, not determined.

^aSI: $\text{CC}_{50}/\text{EC}_{50}$ obtained after 96 h of drug exposure.

^bSoeiro et al. (2013).

^cSI: $\text{CC}_{50}/\text{EC}_{50}$ obtained after 48 h of drug exposure.

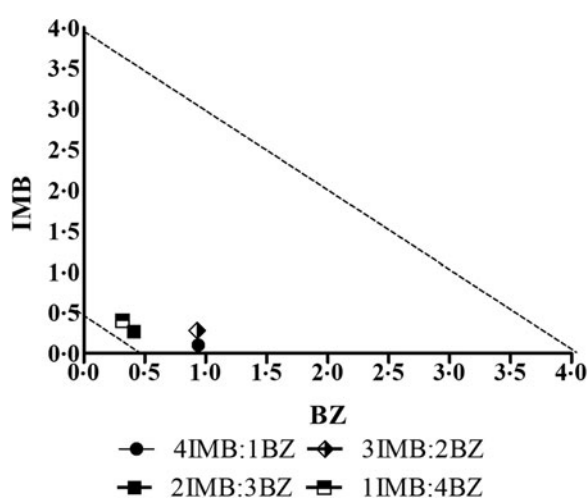
^dExtracellular amastigotes obtained from the supernatant of infected cardiac cells.

activity ($\text{EC}_{50} = 24.8 \pm 7.4 \mu\text{M}$) in comparison to BZ ($\text{EC}_{50} = 4.1 \pm 1.3 \mu\text{M}$). When mammalian cells viability was evaluated, IMB exhibited higher toxicity than BZ after 96 h of incubation ($\text{CC}_{50} = 38.3 \pm 0.2$ and $>50 \mu\text{M}$, respectively) (Table 1). To expand the analysis to other parasite strains and other mammalian host cells, IMB was screened against intracellular forms of the Y strain (DTU II) inside cc, besides evaluating its potential cardiotoxicity profile. After 48 h of drug exposure, IMB and BZ were not toxic up to $50 \mu\text{M}$ (Table 1). The readout of Giemsa-stained *T. cruzi*-infected cc by light microscopy showed the trypanocidal activity of IMB, being able to reduce both the percentage of infected host cells as well as the number of parasites per infected cell (data not shown). Regarding the infection index, the EC_{50} values for IMB and BZ were 20.0 ± 4.4 and $2.8 \pm 1.9 \mu\text{M}$, respectively (Table 1). Aiming to validate the trypanocidal activity of IMB directly against amastigotes without the possible drug influence on the mammalian host cell machinery, free parasites released from a highly infected cardiac culture were incubated also for 48 h. The results confirmed the *in vitro* moderate effect of IMB, reaching EC_{50} values of $30.0 \pm 4.4 \mu\text{M}$ (Table 1).

Table 2. *In vitro* trypanocidal activity (EC₅₀ and EC₉₀ – μM) of benznidazole and imatinib against bloodstream (BT), culture-derived (CT) trypomastigotes from *Trypanosoma cruzi* Y strain, cytotoxicity on cardiac cells (CC₅₀ – μM) and the corresponding selectivity indexes (SI^a)

Time of drug exposure	Benznidazole		Imatinib	
	Mean ± s.d.			
	EC ₅₀ (SI)	EC ₉₀	EC ₅₀ (SI)	EC ₉₀
BT 2 h	>50 (nd)	>50	47.1 ± 9.5 (>1.1)	>50
BT 24 h	14.4 ± 3.4 (>3.5)	22.8 ± 7.8	33.6 ± 4.9 (>1.5)	45.7 ± 0.7
CT 24 h	nd	nd	43.3 ± 19.0 (>1.1)	>50
Cardiac cells 24 h	CC ₅₀			
	>50		>50	

nd, not determined.

^aSI: CC₅₀/EC₅₀ obtained after 24 h of drug exposure.**Fig. 3.** Isobologram against intracellular forms of *Trypanosoma cruzi* Tulahuen strain in L929 cell line. The EC₅₀ of each compound is plotted on the abscissa and the ordinate, respectively. \sum FICs ≤ 0.5 = synergism; 0.5 < \sum FICs ≤ 4.0 = additive (no interaction); \sum FICs > 4.0 = antagonism.

IMB was assayed against CT and BT of the Y strain and the data showed EC₅₀ values of 43.3 ± 19.0 and 33.6 ± 4.9 μM for CT and BT, respectively, after 24 h of incubation (Table 2).

Since BZ and IMB present distinct modes of action, which means acting on different targets, IMB was combined with BZ for drug interaction assays with fixed-ratio and serially diluted concentrations (Fig. 3, Table 3). The isobologram shows that the interaction between both drugs was additive (mean \sum FICs = 0.91) (Fig. 3), and all the \sum FIC for each combination ratio demonstrated that none of them was synergistic (Table 3). The best non-toxic ratio was two parts of IMB to three parts of BZ, with the \sum FIC = 0.68 (Table 3). Toxicity upon L929 cell lines was only observed for the highest concentrations, reaching 91% of loss of cellular viability for 200 μM IMB + 4.8 μM BZ (ratio 4:1, data not shown).

The moderate activity of IMB against different forms and strains of *T. cruzi* motivated the next step performing the phenotypic screening of 14 new analogues. The compounds (Fig. 2) were assayed under the same *in vitro* established models above reported. The toxicity assessment against L929 cell line cultures demonstrated that 1a, 1b, 2c, 3a and 3d were not toxic up to 50 μM after 96 h of incubation (Table 4). However, the other compounds displayed distinct levels of mammalian cells toxicity ranging from 8 to 48 μM (Table 4). When the derivatives were

Table 3. Mean of \sum FICs of interaction between imatinib (IMB) and benznidazole (BZ) on intracellular forms of *Trypanosoma cruzi* Tulahuen strain in L929 cell line

IMB + BZ drug proportion	EC ₅₀ μM		
	FIC – IMB	FIC – BZ	\sum FIC
4 + 1	0.94 ± 0.24	0.10 ± 0.02	1.04
3 + 2	0.93 ± 0.04	0.28 ± 0.07	1.21
2 + 3	0.41 ± 0.12	0.27 ± 0.08	0.68
1 + 4	0.32 ± 0.00	0.4 ± 0.00	0.72
Mean \sum FICs in combination	0.65	0.26	0.91

Top drug concentrations: 4 + 1 = 200 μM of IMB plus 4.8 μM of BZ; 3 + 2 = 150 μM of IMB plus 9.6 μM of BZ; 2 + 3 = 100 μM of IMB plus 14.4 μM of BZ; 1 + 4 = 50 μM of IMB plus 19.2 μM of BZ

screened using L929-infected cc (Tulahuen-β galactosidase strain), ten derivatives, listing 1a, 2a–e, 3a and 3c–e, presented considerable activity, with EC₅₀ values below 10 μM (Table 4). The derivatives 1a, 2d and 3c were most potent (EC₅₀ ≤ 3.8 μM) than BZ, being at least 6-fold more active than IMB (Tables 1 and 4, P ≤ 0.05).

The toxicity assays performed on cc for 24 h revealed that none of the derivatives was cardiotoxic up to 50 μM (Table 4). Then, the ten compounds active against intracellular forms (Tulahuen strain) were tested against BT (Y strain). Compounds 1a, 2a, 2c, 2e, 3a and 3c showed no trypanocidal activity up to 50 μM (Table 4). The best trypanocidal molecule was 3e (EC₅₀ = 7.4 μM), being 5-fold more active than IMB (P ≤ 0.05) (Tables 2 and 4). This derivative investigated on *T. cruzi*-infected cc (Y strain) displayed EC₅₀ values of 1.3 ± 0.4 μM with SI >38 (Table 5), in the same activity range of BZ (Table 1).

Discussion

The focus of the study was to access the phenotypic profile of IMB and the 14 novel derivatives, by using different strategies recommended for the drug discovery of CD: drug repurposing and combination, thus aiming to contribute for the identification of novel therapies more specific towards the parasite and less dangerous to mammalian cells.

IMB is a TK inhibitor with high selectivity for ABL kinases, mostly present in tumourigenic cells (Cruz-Rico *et al.*, 2013; Musumeci *et al.*, 2015). The activity of IMB has also been demonstrated against several pathogens, including helminths (Beckmann *et al.*, 2014; Buro *et al.*, 2014; O'Connell *et al.*, 2015) and protozoa

Table 4. *In vitro* trypanocidal activity (EC₅₀) of imatinib derivatives against intracellular forms (Tulahuen strain, 96 h of drug exposure) and bloodstream trypomastigotes (Y strain, 24 h of drug exposure), as well as the cytotoxic concentration for mammalian cells (CC₅₀, 24 and 96 h) and the corresponding selectivity index (SI)

Compounds	CC ₅₀ L929	EC ₅₀ Tulahuen/L929	SI ^a	CC ₅₀ CC	EC ₅₀ BT	SI ^b
1a	>50	3.8 ± 1.5	>13.2	>50	>50	nd
1b	>50	>50	nd	>50	nt	nd
1c	39.8 ± 0.8	30.5 ± 7.7	1.3	>50	nt	nd
1e	36.9 ± 3.2	11.6 ± 8	3.2	>50	nt	nd
2a	31.6 ± 1.1	6.8 ± 0.8	4.6	>50	>50	nd
2b	20.4 ± 5.1	5.7 ± 0.3	3.6	>50	39.2 ± 5.1	>1.3
2c	>50	6.7 ± 2.7	>7.5	>50	>50	nd
2d	8.3 ± 6.5	3.1 ± 0.6	2.7	>50	29.8 ± 5.0	>1.7
2e	11.9 ± 0.5	5.7 ± 0.6	2.1	>50	>50	nd
3a	>50	6.1 ± 2.5	>8.2	>50	>50	nd
3b	47.8 ± 0.3	27.4 ± 7.7	1.7	>50	nt	nd
3c	39.6 ± 9.6	2.0 ± 1.1	18.5	>50	>50	nd
3d	>50	6.1 ± 0.8	>8.2	>50	11.1 ± 0.6	>4.5
3e	14.3 ± 11.7	4.9 ± 1.9	2.9	>50	7.4 ± 0.7	>6.8

nt, not tested; nd, not determined.

^aSI: CC₅₀/EC₅₀ obtained after 96 h of drug exposure.

^bSI: CC₅₀/EC₅₀ obtained after 24 h of drug exposure.

Table 5. *In vitro* trypanocidal activity (EC₅₀ and EC₉₀ – μM) of **3e**, an imatinib derivative, against intracellular amastigote forms of the Y strain of *Trypanosoma cruzi* in mice primary cardiac cell culture and selectivity index (SI)

3e	EC ₅₀ (SI) ^a	EC ₉₀
Mean ± s.d. μM	1.3 ± 0.4 (>38.4)	>50

^aSI: CC₅₀/EC₅₀ obtained after 48 h of drug exposure.

(Wetzel *et al.*, 2012; Behera *et al.*, 2014; Pathak *et al.*, 2015), suggesting the repurposing potential of this drug.

As suggested for CD flowchart screenings (Romanha *et al.*, 2010), the toxicity profile of IMB and derivatives was explored using different host cells (primary cardiac cells and fibroblasts of L929 cell lines). The trypanocidal effect was investigated upon the relevant forms of the parasite for mammalian infection (amastigotes and trypomastigotes) (Brenner and Chiari, 1963) obtained from different sources (culture-derived and BT, intracellular and extracellular amastigotes) and from distinct strains, which are susceptible (Tulahuen strain) and the partially naturally resistant to nitroderivatives (Y strain) belonging to different discrete typing units – DTUS VI and II, respectively (Zingales *et al.*, 2012).

Our present results demonstrate the moderate activity of IMB against *T. cruzi* *in vitro* using different parasite strains and forms, corroborating previous studies reported against strains obtained from chronic chagasic patients (CA-I/72 clone and PSD-1), and clones derived from insect (Sylvio-X10/7 clone and its parental strain) (Engel *et al.*, 2010; Dichiaro *et al.*, 2017). Analogues of TK are present in protozoa such as trypanosomatids, including *T. cruzi*, and have been related to several parasite metabolic processes besides playing a role during host cell invasion (Melo *et al.*, 2014). IMB was moderately active against *Plasmodium falciparum* (Pathak *et al.*, 2015) and *Leishmania amazonensis* (Wetzel *et al.*, 2012), being the enzyme inhibition demonstrated. However, IMB was inactive against *Trypanosoma brucei gambiense*, although some of its analogues showed mild potency, especially lapatinib

(Behera *et al.*, 2014), which was used as scaffold for new molecules further tested against *L. major*, *T. brucei* and *T. cruzi* with promising results (De Rycker *et al.*, 2016; Devine *et al.*, 2017).

In our studies, besides assessing the trypanocidal activity of IMB, 14 novel derivatives were studied using well-established *in vitro* methodologies for drug screening in *T. cruzi* (Romanha *et al.*, 2010). These new derivatives have the phenylamino-pyrimidine group as their main pharmacophore fragment and presented antitumour activity *in vitro* (Moreno *et al.*, 2010). The series **1a–e** has hybrid molecules developed from IMB and sunitinib, another TK inhibitor. The molecules of series **2a–e** have 2-oxo-2-phenylacetamides, the ones of series **3a–e** have 3,2-difluoro-2-phenylacetamides, and isatins were used as starting molecules in all series (Azevedo *et al.*, 2017).

The TDR (Special Programme for Research and Training in Tropical Diseases, World Health Organization) establishes criteria for the determination of antiparasitic activity and selectivity of compounds in test: EC₅₀ lower than 4.0 μM are 'active', between 4.0 and 60 μM are 'moderately active' and higher than 60 μM are 'inactive' against *T. cruzi* amastigotes (Papadopoulou *et al.*, 2015). Accordingly, although IMB presented a moderate antiparasitic activity (against different parasite forms and strains of *T. cruzi*), with EC₅₀ values 4- to 6-fold higher than the reference drug (BZ). Some of the studied derivatives displayed an enhanced anti-*T. cruzi* effect, being active in micromolar range, which represents a dramatic improvement in the trypanocidal activity in relation to the scaffold molecule already reported in the literature (Engel *et al.*, 2010). The compounds **1a**, **2a–e**, **3a** and **3c–e** presented trypanocidal effects under 10 μM against intracellular forms of the Tulahuen strain, being more potent than IMB (EC₅₀ = 38.3 μM) and **3d** and **3e** exhibited superior/similar effect as BZ when assayed against BT. However, poor selectivity was achieved for most of them.

Moving forward, as combined therapy has been largely used for different diseases with promising results (Sun *et al.*, 2016), we investigated the trypanocidal effect of the IMB plus BZ. Although according to the sum of FICs (ΣFICs), the combination was additive, the FIC values differed among the fixed-ratio

proportions suggesting that a combination of two parts of IMB to three parts of BZ might have a better predictive *in vivo* outcome than the monotherapies. Another interesting feature was the low toxicity profile of the combination IMB + BZ. L929 cellular viability dropped by 91% only when higher concentrations of IMB were used, such as 200 μM IMB + 4.4 μM BZ. The strategy of combining compounds acting on distinct targets is promising as reported for other drug combinations (Simões-Silva *et al.*, 2016; Santos *et al.*, 2018), improving parasite killing. In this sense, approaches for the synthesis and development of novel IMB derivatives using possibly a hybrid molecule composed by IMB derivatives (such as compound 3e) and BZ could be performed aiming to improve the activity and selectivity of these compounds as anti-*T. cruzi* agents.

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Ethical standards. Swiss mice (21–23 g) were provided by the Instituto de Ciência e Tecnologia em Biomodelos (ICTB/Fiocruz) (Rio de Janeiro, Brazil). They were kept in a conventional room at 20–24 °C under a 12/12 h light/dark cycle. The animals were supplied with sterilized water and chow *ad libitum*. The procedures complied to the guidelines of the FIOCRUZ Committee of Ethics for the Use of Animals (CEUA LW16/14).

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