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

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**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.



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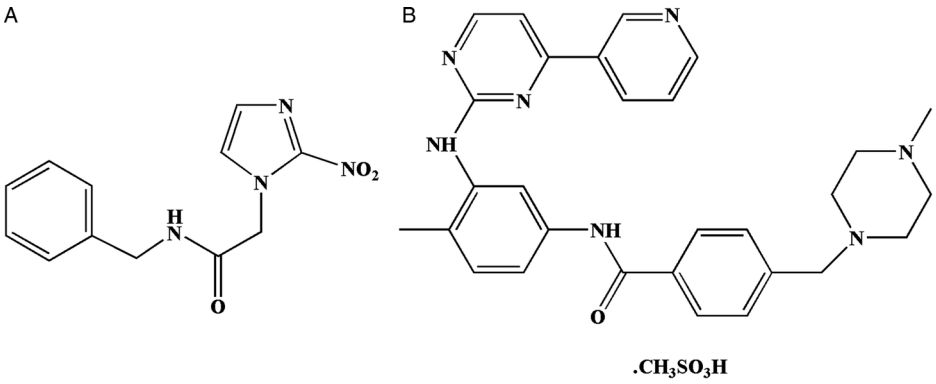


**Fig. 1.** Chemical structures of benznidazole (**A**) and imatinib mesylate (**B**).

## 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 $\mu$m as some of the derivative molecules slightly precipitated (light microscopy observation) using higher concentrations.

### Mammalian cell cultures

Mouse fibroblasts from L929 cell line ($4 \times 10^3$ 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 $\beta$-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 $\mu$m) 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 $LC_{50}$ (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 $CC_{50}$ for host cells, and the $EC_{50}$ for the parasites or infected cc (SI: $CC_{50}/EC_{50}$). 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 ($5 \times 10^6$ mL$^{-1}$) were incubated for 2 and 24 h at 37 °C in RPMI with 1:3 serial dilutions of the compounds (0–50 $\mu$m) for determination of parasite death rates, through the direct quantification of live parasites by light microscopy. The $EC_{50}$ (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 $\mu$m) in RPMI. After the incubation, 500 $\mu$M chlorophenol red-$\beta$-D-galactopyranoside in 0.5% Nonidet P40 was added to each well (final concentration 50 $\mu$m) 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 $EC_{50}$ 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 $\mu$m), 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 $EC_{50}$ 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, $5 \times 10^6$ parasites mL$^{-1}$ were incubated for 48 h at 37 °C with increasing concentrations of the studied compounds

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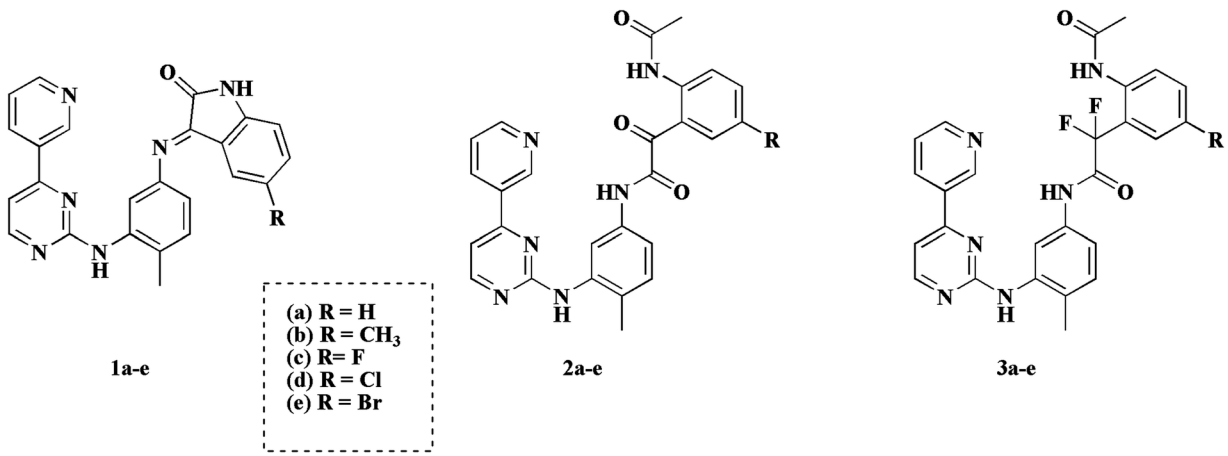


**Fig. 2.** Chemical structures of imatinib derivatives.

(⩽50 $\mu$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 $\beta$-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 ($\sum$FICs) were calculated as follows: FIC of IMB = $EC_{50}$ of IMB in combination/$EC_{50}$ of IMB in monotherapy. The same equation was applied to the partner drug BZ. The $\sum FICs = FIC_{IMB} + FIC_{BZ}$. An overall $\sum$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). $\sum FICs \leqslant 0.5$ = synergism; $0.5 < \sum FICs \leqslant 4.0$ = additive (no interaction); $\sum FICs > 4.0$ = 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 $P \leqslant 0.05$ 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 $\mu$M) and readout using colorimetric analysis. As presented in Table 1, IMB displayed a moderate trypanocidal activity ($EC_{50}$ = 24.8 ± 7.4 $\mu$m) in comparison to BZ ($EC_{50}$ = 4.1 ± 1.3 $\mu$m). When mammalian cells viability was evaluated, IMB exhibited higher toxicity than BZ after 96 h of incubation ($CC_{50}$ = 38.3 ± 0.2 and >50 $\mu$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 $\mu$m (Table 1). The readout of Giemsa-stained *T. cruzi*-infected cc by light microscopy showed the trypanocidality 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 ± 1.9 $\mu$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 ± 4.4 $\mu$M (Table 1).

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

| | Mean ± S.D. | |
|---|---|---|
| *T. cruzi* amastigote forms (strains and sources) and mammalian cells | Benznidazole | Imatinib |
| $CC_{50}$ 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.
[a]SI: $CC_{50}/EC_{50}$ obtained after 96 h of drug exposure.
[b]Soeiro *et al.* (2013).
[c]SI: $CC_{50}/EC_{50}$ obtained after 48 h of drug exposure.
[d]Extracellular amastigotes obtained from the supernatant of infected cardiac cells.

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**Table 2.** *In vitro* trypanocidal activity ($EC_{50}$ and $EC_{90}$ – $\mu$M) of benznidazole and imatinib against bloodstream (BT), culture-derived (CT) trypomastigotes from *Trypanosoma cruzi* Y strain, cytotoxicity on cardiac cells ($CC_{50}$ – $\mu$M) and the corresponding selectivity indexes (SI[a])

| | Benznidazole | | Imatinib | |
|---|---|---|---|---|
| | Mean ± S.D. | | | |
| Time of drug exposure | $EC_{50}$ (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.
[a]SI: CC50/EC50 obtained after 24 h of drug exposure.

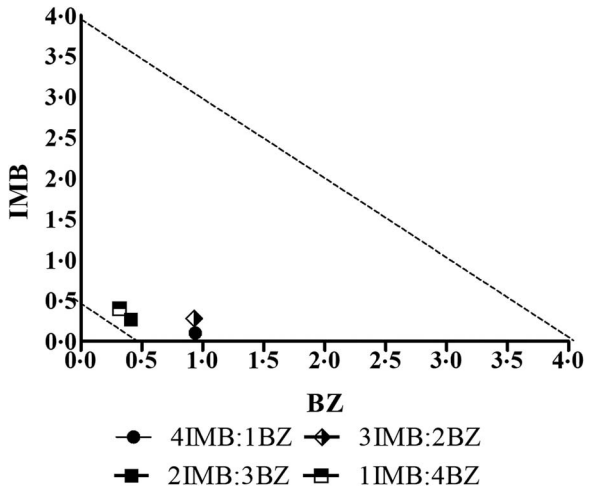


**Fig. 3.** Isobologram against intracellular forms of *Trypanosoma cruzi* Tulahuen strain in L929 cell line. The $EC_{50}$ of each compound is plotted on the abscissa and the ordinate, respectively. $\sum$FICs ⩽ 0.5 = synergism; 0.5 < $\sum$FICs ⩽ 4.0 = additive (no interaction); $\sum$FICs < 4.0 = antagonism.

**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

| | $EC_{50}$ $\mu$M | | |
|---|---|---|---|
| IMB + BZ drug proportion | 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 $\mu$m of IMB plus 4.8 $\mu$m of BZ; 3 + 2 = 150 $\mu$m of IMB plus 9.6 $\mu$m of BZ; 2 + 3 = 100 $\mu$m of IMB plus 14.4 $\mu$m of BZ; 1 + 4 = 50 $\mu$m of IMB plus 19.2 $\mu$m of BZ

IMB was assayed against CT and BT of the Y strain and the data showed $EC_{50}$ values of 43.3 ± 19.0 and 33.6 ± 4.9 $\mu$m 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 $\mu$m IMB + 4.8 $\mu$m 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 $\mu$m after 96 h of incubation (Table 4). However, the other compounds displayed distinct levels of mammalian cells toxicity ranging from 8 to 48 $\mu$m (Table 4). When the derivatives were screened using L929-infected cc (Tulahuen-$\beta$ galactosidase strain), ten derivatives, listing **1a, 2a–e, 3a** and **3c–e**, presented considerable activity, with $EC_{50}$ values below 10 $\mu$m (Table 4). The derivatives **1a, 2d** and **3c** were most potent ($EC_{50}$ ⩽ 3.8 $\mu$m) than BZ, being at least 6-fold more active than IMB (Tables 1 and 4, $P \leqslant 0.05$).

The toxicity assays performed on cc for 24 h revealed that none of the derivatives was cardiotoxic up to 50 $\mu$m (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 $\mu$M (Table 4). The best trypanocidal molecule was **3e** ($EC_{50}$ = 7.4 $\mu$m), being 5-fold more active than IMB ($P \leqslant 0.05$) (Tables 2 and 4). This derivative investigated on *T. cruzi*-infected cc (Y strain) displayed $EC_{50}$ values of 1.3 ± 0.4 $\mu$m 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

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**Table 4.** *In vitro* trypanocidal activity ($EC_{50}$) 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 ($CC_{50}$, 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.
[a]SI: $CC_{50}/EC_{50}$ obtained after 96 h of drug exposure.
[b]SI: $CC_{50}/EC_{50}$ obtained after 24 h of drug exposure .

**Table 5.** *In vitro* trypanocidal activity ($EC_{50}$ and $EC_{90}$ – $\mu$M) 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** Mean ± S.D. $\mu$M | $EC_{50}$ (SI)[a] | $EC_{90}$ |
|---|---|---|
| | 1.3 ± 0.4 (>38.4) | >50 |

[a]SI: $CC_{50}/EC_{50}$ 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) (Brener 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; Dichiara *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 phenylaminopyrimidine 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: $EC_{50}$ lower than 4.0 $\mu$M are 'active', between 4.0 and 60 $\mu$m are 'moderately active' and higher than 60 $\mu$M 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 $EC_{50}$ 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 $\mu$M against intracellular forms of the Tulahuen strain, being more potent than IMB ($EC_{50}$ = 38.3 $\mu$m) 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 ($\sum$FICs), the combination was additive, the FIC values differed among the fixed-ratio

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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 $\mu$m IMB + 4.4 $\mu$m 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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**Conflict of interest.** None.

**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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