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ABSTRACT

New affordable drugs are needed for the treatment of infection with the protozoan parasite *Trypanosoma cruzi* responsible for the Chagas disease (CD). Only two old drugs are currently available, nifurtimox and benznidazole (Bz) but they exhibit unwanted side effects and display a weak activity in the late chronic phase of the disease. In this context, we evaluated the activity of a series of aryl-pyrazolone derivatives against *T cruzi*, using both bloodstream trypomastigote and intracellular amastigote forms of the parasite. The test compounds originate from a series of anticancer agents targeting the immune checkpoint ligand PD-L1 and bear an analogy with known anti-trypanosomal pyrazolones. A first group of 6 phenyl-pyrazolones was tested, revealing the activity of a single pyridyl-pyrazolone derivative. Then a second group of 8 compounds with a common pyridyl-pyrazolone core was evaluated. The *in vitro* testing process led to the identification of two non-cytotoxic and highly potent molecules against the intracellular form of *T. cruzi*, with an activity comparable to Bz. Moreover, one compound revealed an activity largely superior to that of Bz against bloodstream trypomastigotes, while being non-cytotoxic (selectivity index >1000). Unfortunately, the compound showed little activity *in vivo*, most likely due to its very limited plasma stability. However, the study opens novel perspectives for the design of new anti-trypanosomal products and the mechanism of action of the compounds is discussed.

1. Introduction

Chagas disease (CD) caused by the protozoan parasite *Trypanosoma cruzi* is one of the most important neglected tropical diseases in the Americas. It is also present in Europe, Japan, and Australia due to populational migration (Abras et al., 2022; Hochberg and Montgomery, 2023). CD remains endemic in vast areas, despite the existence of two approved drugs. Benznidazole (Bz) and nifurtimox are used for more than 60 years but they have important limitations and can induce serious adverse effects. Notably, they exhibit a low efficacy at the late chronic stage of the disease and induce multiple adverse reactions such as dermatitis, leuco/neutropenia and other responsible for high drop-out rates (Gontijo et al., 2020). According to the World Health Organization (WHO), more than 6 million people worldwide are inflicted by Chagas disease and about 75 million are under risk of infection (WHO, 2023). Fewer than 10% of infected people are diagnosed and even fewer are effectively treated (DNDi, 2023). With no tangible progress toward new therapies for CD for more than 50 years, the search for new active and safe drugs for CD remains a priority (Kratz et al., 2022; Tarleton, 2023; De Rycker et al., 2023).

In recent years, few drug candidates have been tested in clinical trials of CD, including two inhibitors of CYP51 (sterol 14α-demethylase) posaconazole and the water-soluble prodrug of ravuconazole know as E1224 or fosravuconazole (Torrico et al., 2018; Mazzeti et al., 2021).

Abbreviations: CD, Chagas disease; PD-L1, programmed death-ligand 1; TcrPDEC1, *T. cruzi* phosphodiesterase C1.

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Recently, the 5-nitroimidazole derivative fexinidazole has been approved for the treatment of African trypanosomiasis (cause by *T. brucei gambiense*) and it is in development for CD (Pfarr et al., 2023; Soeiro, 2022). Azole drugs are interesting but they are not able to induce long-term durable responses in chronically infected CD patients (Molina et al., 2014). Unlike benznidazole, E1224 was shown to display a transient, suppressive effect on *T. cruzi* clearance (Torrico et al., 2018). In a recent proof-of-concept phase 2 trial, fexinidazole was shown to induce a sustained parasitological clearance but it induced delayed-onset neutropenia and increased liver enzymes. Further evaluation of this drug is required to better determine the minimum effective dosage and risk-benefit relationships (Torrico et al., 2023). There are also a few new experimental drugs, such as the promising prodrug AN15368 that will be soon evaluated in CD patients (Padilla et al., 2022), in addition to drug repurposing strategies (Porta et al., 2023; Galceran et al., 2023).

The development of new drugs for CD is very challenging, notably due to the lack of validated targets. The importance of experimental models has been underlined also, notably the need for models that can address both the rapidly-dividing and non-dividing (quiescent) forms of the parasites. Tackling the parasites which remain dormant or as persisters is essential (De Rycker, 2022; 2023). New models and novel therapeutic approaches are needed (Soeiro, 2022; Gabaldón-Figueira et al., 2023). There are innovative novel strategies to target parasite essential proteins such as cruzain, trypanothione reductase, CPSF3 and a few others (Laureano de Souza et al., 2023).

T. cruzi infection leads to an unbalanced inflammatory response associated to a dysregulated immune mechanism (Dutra et al., 2014). The pathogen activates immune response via a process involving inflammasome and virulence factors. An ineffective immunological response can increase the parasitic load sustaining its persistence, generally associated with an excessive inflammatory response which causes tissue damages (Macaluso et al., 2023). Antigen-specific T cells are central to the adaptive immune response against *T. cruzi* infection (Ferragut et al., 2023). Notably, the immune response against *T. cruzi* substantially relies on the efficiency of both $CD4^+$ and $CD8^+$ T cells to control parasite growth (Biscari et al., 2022). In this frame, it has been shown that a long-term chronic murine infection leads to dysfunctional $CD8^+$ T cells, characterized by a high expression of the inhibitory receptor PD-1 (Gálvez and Jacobs, 2022).

Targeting of the PD-1/PD-L1 checkpoint (programmed cell death protein 1/programmed death-ligand 1) has revolutionized the treatment of cancers (at least for certain types of solid tumors), via the development of monoclonal antibodies targeting either PD-1 or PD-L1 to reinvigorate exhausted T cells in the tumor microenvironment (Ai et al., 2020). Anti-PD-1 and anti-PD-L1 monoclonal antibodies have been tested also in different parasitic diseases, such as Leishmaniasis, with the objective to promote the reinvigoration of T cells for the control of parasite burden (da Fonseca-Martins et al., 2019, 2021). PD-1/PD-L1 blockade is considered as a potential therapeutic strategy to restore protective immunity during leishmaniasis (Jafarzadeh et al., 2022).

The situation is more controversial in cases of experimental *T. cruzi* infection. Blockade of PD-1 has been shown to reduce parasitemia and tissue parasitism, but paradoxically it increased mortality. It seems that the PD-1/PD-L1 signaling pathway is implicated in the control of acute myocarditis induced by *T. cruzi* (Gutierrez et al., 2011). On the other hand, recent data indicated that the interruption of the PD-1/PD-L1 axis during acute murine *T. cruzi* infection does not necessarily enhance the immune response against this parasite since it rather favors a higher parasitemia and does not improve the outcome. Also, therapy using an anti-PD-1 (combined with an anti-TIM-3) had no significant effect on parasitemia *in vivo* (Arana et al., 2022).

Beyond monoclonal antibodies, a panoply of small molecules targeting PD-L1 have been designed and a few of them are currently evaluated in the clinic for the treatment of cancers, such as INCB086550 (Kopalli et al., 2019; Li and Tian, 2019; Koblish et al., 2022). Orally available small molecules could be extremely useful to treat cancers, offering convenient alternatives to antibody-based therapies, notably to avoid the immune-related adverse effects of antibodies (Hao et al., 2023). Our medicinal chemistry efforts in this domain have led to the design of a series of phenyl-pyrazolone derivatives acting as PD-L1 silencing compounds, with nanomolar affinity for the PD-L1 protein dimer (Le Biannic et al., 2022). Interestingly, these molecules not only target PD-L1 but they also display antioxidant properties and a reactivity toward aromatic aldehydes (Leleu-Chavain et al., 2022). The phenyl-pyrazolone unit functions as a scavenger of oxygen free radicals, according to a mechanism well elucidated for the structurally related drug edaravone, which is used for the treatment of the fatal neurodegenerative disorder amyotrophic lateral sclerosis (Cho and Shukla, 2020; Pattee et al., 2023). Our phenyl-pyrazolone compounds are potent free radical scavenging (antioxidant) agents and have the capacity to react with different aldehydes (such as 4-hydroxynonenal), in addition to their anti-PD-1 activity (Regnault et al., 2023). Considering that the defense against oxidative stress plays a role against parasitic diseases (Santi and Murta, 2022) and that antioxidant compounds can be useful to reduce damages caused by *T. cruzi*-induced hepatic oxidative stress (Sánchez-Villamil et al., 2020; Freitas et al., 2020; Fracasso et al., 2021), we decided to investigate the potential anti-trypanosomal activity of a selected sub-group of phenyl-pyrazolone derivatives. The work was also oriented by previous studies which have revealed that phenyl-pyrazolone monomers and dimers can display marked anti-trypanosomal activities, such as the PDE inhibitor named NPD-0227 that was potently active *in vitro* against intracellular forms of *T. cruzi* (Sijm et al., 2019, 2020, 2021; de Araújo et al., 2020a).

In a first instance, 6 phenyl-pyrazolone compounds were selected, including one phenyl-pyrazolone (**1**), two diphenyl-pyrazolone (**2** and **3**), a phenyl-pyrazolone bearing an adamantane unit (**4**), a phenyl-pyrazolone dimer (**5**) and a pyridine-pyrazolone derivative (**6**) (Fig. 1a). Most of these molecules are robust PD-L1 inhibitors, with nanomolar affinity for human PD-L1. For examples, compounds **1**, **2**, **4** and **6** avidly bind to PD-L1, inducing the dimerization of the protein. They disrupt the PD-L1/PD-1 interaction in mammalian cells, so as to deactivate the downstream signaling, notably the activity of the tyrosine phosphatase SHP-2, as shown previously using a specific FRET assay (Table 1). After a preliminary evaluation, the pyridine-type compound **6** was tested further against *T. cruzi* together with 8 derivatives, all including a pyridyl-pyrazolone core (Fig. 1b). This is the first study investigating the anti-trypanosomal activity of small synthetic molecules targeting PD-L1.

2. Materials and methods

2.1. Compounds and chemicals

The synthesis of the diverse phenyl- and pyridine-pyrazolone derivatives has been previously described (Le Biannic et al., 2022; Leleu-Chavain et al., 2022) and their use as PD-L1 inhibitors has been patented (Thuru et al.). An improved synthetic procedure has been reported recently (Regnault et al., 2023).

The synthesis of compound **1** is shown in Scheme 1. To a solution of diethyl 1,3-acetonedicarboxylate (1.2 eq, 2.71 mmol) in EtOH (20 mL) were added (2,4-dichlorophenyl)hydrazine hydrochloride (1 eq, 2.26 mmol) and TEA (1 eq, 2.26 mmol) and the resulting mixture was brought to reflux temperature for 2 h. After reaction, the solvent was evaporated under reduced pressure. The reaction medium was poured into 100 ml of water. The residue was extracted with ethyl acetate, and then washed with brine. The organic layer was dried over $MgSO_4$ and evaporated under reduced pressure. A quantity of 680 mg of diethyl 3-(2-(2,4-dichlorophenyl)hydrazineylidene)pentanedioate was obtained and use subsequently without purification. Rf (cyclohexane/ethyl acetate 7:3): 0.66. ^{1}H NMR ($CDCl_3$) δ 8.78 (s,1H); 7.41 (d, $J = 8.8$ Hz, 1H); 7.30 (d, $J = 2.3$ Hz, 1H); 7.18 (Dd, $J = 8.8$ Hz, J' = 2.3 Hz, 1H); 4.23 (q, $J = 7.1$ Hz, 2H); 3.53 (s, 2H); 2,48 (s, 2H); 1.33 (t, $J = 7.1$ Hz, 3H). LC-

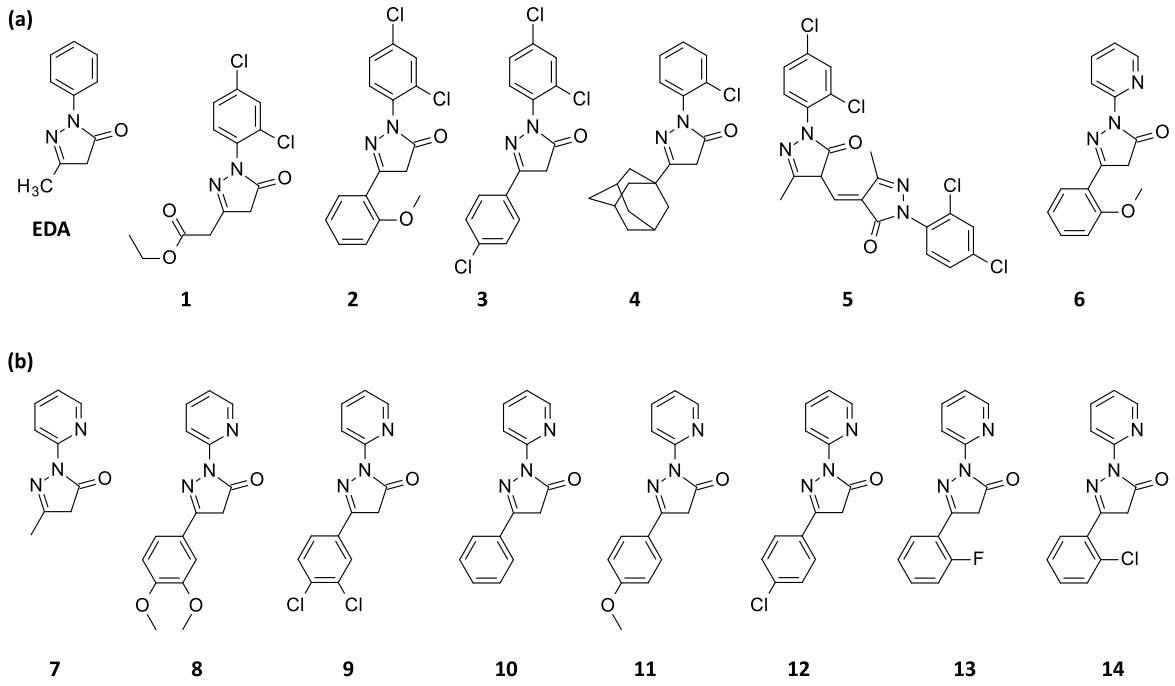


Fig. 1. Structure of the studied compounds. (a) 1st series, (b) 2nd series of pyridyl-pyrazolone.

Table 1
PD-L1 binding and cell activity.

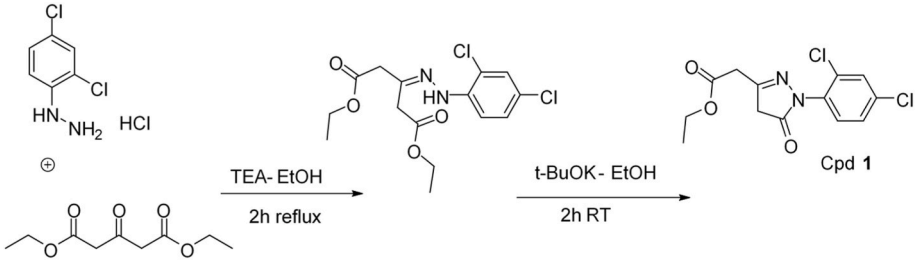
Compounds #	PD-L1 binding (MST assay)[a]	PD-L1 signaling (FRET assay)[b]
	K_D (nM)	IC_{50} (nM)
1	0.59 ± 0.12	27 ± 12
2	45 ± 7	44 ± 9
3	7 ± 3	3 ± 2
4	12 ± 2	23 ± 6
5	173 ± 34	45 ± 8
6	23 ± 5	7 ± 4
7	77 ± 7	92 ± 9
8	1.19 ± 0.4	7.30 ± 1.20
9	584 ± 75	–
10	385 ± 21	359 ± 17
11	83 ± 12	17 ± 5
12	n.t.	n.t.
13	n.b.	–
14	n.b.	–

[a] Affinity for human recombinant PD-L1 measured by microscale thermophoresis (MST) (Magnez et al., 2017).

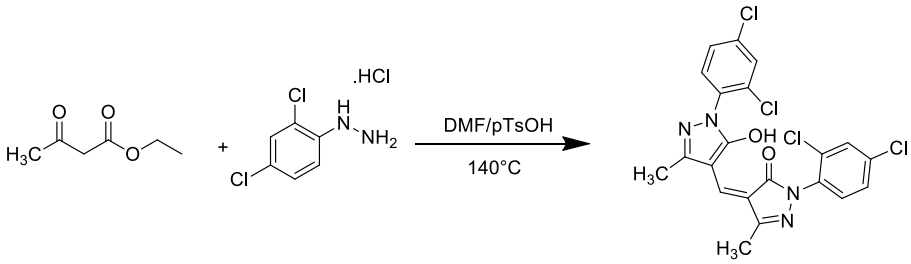
[b] Capacity of the compounds to disrupt the PD-L1/PD-1 interaction in engineered CHO–K1 cells (to express the fluorescent fusion proteins SHP-2-CFP and PD-1-YFP), measured by a fluorescence resonance energy transfer (FRET) assay. The tests and methods have been previously described (Le Biannic et al., 2022; Leleu-Chavain et al., 2022; Thuru et al., 2023). The compounds showing no-binding (n.b.) or a weak binding capacity in the MST assay were not tested in the FRET assay (-). Not tested (n.t.).

MS (ESI) m/z 359 (MH-), tr 2.55 min. In a balloon of 50 ml equipped with a condenser, diethyl 3-(2-(2,4-dichlorophenyl) hydrazineylidene) (1 eq, 650 mg, 1.8 mmol) was diluted in EtOH (5 mL) under magnetic stirring at 20 °C. KOtBu (1 eq, 1.8 mmol, 1.8 ml solution 1M in THF) was added and the reaction mixture was stirred at room temperature for 2 h. The solvent was removed in vacuo. The residue was taken up in water (25 mL), and then acidified with glacial acetic acid. The precipitate was filtered, washed with water and dried under vacuum. The product was recrystallized with heptane. Cream colored solid. Yield 70%. Rf (cyclohexane/ethyl acetate 7:3): 0.23. ^{1}H NMR (DMSO): δ 11.37 (s, 1H); 7.82 (d, J = 2.2 Hz, 1H); 7.55 (Dd, J = 8.5 Hz, J' = 2.2 Hz, 1H); 7.47 (d, J = 8.5 Hz, 1H); 5.45 (s, 2H); 4.10 (q, J = 7.1 Hz, 2H); 5,0.45 (s, 2H); 1.20 (t, J = 7.1 Hz, 3H). LC-MS (ESI) m/z 313 (MH-), tr 2.43 min.

A specific synthetic scheme has been applied for compound **5** (Scheme 2). p-Toluenesulfonic acid monohydrate (p-TsOH.H_2O, 1.0 mmol) was added to a solution of ethyl 3-oxo-3-methylpropanoate (1.0 mmol) and 2,4-dichlorophenyl hydrazine hydrochloride (1.0 mmol) in dry DMF (5.0 mL). The reaction mixture was stirred at 140 °C during 6h. The mixture was cooled to room temperature, quenched with water, and extracted with ethyl acetate. The organic layer was washed with a solution of $NaHCO_3$ (10%) and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel using cyclohexane-ethyl acetate (7-3) as the eluent to give an orange solid. The product was recrystallized with acetonitrile. Yield: 32 %. mp: 234 °C. Rf (cyclohexane/ethyl acetate 6:4): 0,75. ^{1}H NMR (DMSO): 2,39 (s, 6H); 7,59-7,63 (dd, 2H, ^1J = 8.5 Hz, ^2J = 2.0 Hz);



Scheme 1. Synthesis of compound **1**.



Scheme 2. Synthesis of compound **5**.

7.63-7,67 (m, 3H); 7,90 (d, 2H, $J = 1.8$ Hz). LC-MS (ESI^+): 496.9 (MH^+); tr = 3.77 min ^{13}C NMR ($CDCl_3$) δ 162, 153, 139, 136, 133, 131, 130, 128, 108, 13.

Benznidazole (Bz) was used as reference drug and purchased from Laboratório Farmacêutico do Estado de Pernambuco (Brasil). Bz was prepared as stock solution in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, United States) and all aliquots stored at −20 °C. In all *in vitro* and *in vivo* assays, Bz was used as final working concentrations, never exceeding 0.6 % and 10%, which means no toxic effect for cells and animals, respectively (de Araújo et al., 2020a, Ferreira de Almeida Fiuza et al., 2018).

2.2. PD-L1 binding and PD-L1-dependent activity

The capacity of the compounds to bind to recombinant human PD-L1 was assessed by microscale thermophoresis (MST), as previously described (Magnez et al., 2017). Their capacity to interfere with PD-L1 activity in cells and to modulate cellular proliferation has been reported (Le Biannic et al., 2022; Thuru et al., 2023).

2.3. Antiparasitic activity

2.3.1. Mammalian cells

Cell lines L929, H9C2 and HepG2 were routinely maintained through weekly dissociation with 0.01% trypsin solution. The cells were seeded in 96-wells microplates (4×10^3 cells/well) and maintained at 37 °C in RPMI 1640 medium (Sigma-Aldrich, Missouri, USA) supplemented with 10 % FBS, 1 % L-glutamine and 1 % of penicillin. Cell cultures were maintained at 37 °C at 5 % CO_2 as reported (Fiuza et al., 2022; Romanha et al., 2010)

2.3.2. Parasites

Bloodstream trypomastigote forms of *T.cruzi* (Y strain, DTU II) were obtained by cardiac puncture of infected Swiss male mice (Meirelles et al., 1986). The trypomastigote forms of Tulahuen strain (DTU VI) expressing the *E. coli* β-galactosidase gene were collected from the supernatant of L929 cells previously infected (host:parasite cell ratio 10:1) (Romanha et al., 2010). For both strains, purified parasites were added to RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS) to perform assays at 37 °C in 5% CO2.

2.3.3. Cytotoxicity assay

Toxicity towards mammalian cells was analyzed upon serial dilution of each compound (8 concentrations with a 1:2 dilution factor) into uninfected cell cultures. The incubation period was 24 or 96 h at 37 °C in RPMI-1640 medium supplemented with 1% L-glutamine and 10% FBS. Aspects of cell morphology were observed under a light microscope and the cellular viability evaluated using the reagent PrestoBlue™ (for H9C2 and HepG2 cells) or AlamarBlue® (for L929 cells) (Romanha et al., 2010; Santos et al., 2022). The percentage of reduction in the host cell viability was determined using a non-linear regression curve to calculate the CC_{50} value (minimum concentration that reduces cellular viability by 50%).

2.3.4. Anti-T. cruzi activity in vitro

The activity of the compounds against intracellular forms of *T. cruzi* (Tulahuen strain transfected with the β-galactosidase gene) was performed using L929 cells sustained in RPMI's medium (supplemented with 10 % FBS, 2% L-glutamine) (Romanha et al., 2010). The cell line was infected with culture-derived trypomastigotes (parasite:host cell ratio 10:1). After 2 h of interaction, infected cultures were rinsed to remove non-internalized parasites and further incubated for 24 h at 37 °C. The infected cultures were treated for 96 h at 37 °C with the compounds (serially diluted 1:2). Then, 50 μL of the 500 μM chlorophenol red glycoside solution diluted in 0.5% of Nonidet P40 was added, and the infected cultures incubated for 18 h at 37 °C, with readouts performed by spectrophotometry at 570 nm. Benznidazole and DMSO (solvent used for the compounds) were run in parallel as positive and negative controls, respectively. The activity of the compounds was expressed by the EC_{50} and EC_{90} values, which represent the concentration capable of inducing a 50% and 90% loss of parasite viability, respectively (Romanha et al., 2010).

The antiparasitic activity of the compounds against bloodstream trypomastigotes (Y strain) was also investigated. Briefly, 100 μL of a trypomastigote suspension (in RPMI medium + 5% FBS) containing 10^7 parasites/mL was added in 96-well microtiter plates to the same volume of each compound diluted in RPMI + 5 % FBS at twice of the desired final concentration (serially diluted 1:2). After 2 and 24 h at 37 °C, the number of live parasites (identified by their characteristic morphology and movement) was determined by light microscope quantification using a Neubauer chamber (de Araújo et al., 2019) and using PrestoBlue™ (Santos et al., 2022). Untreated controls were carried out with parasites kept under the same conditions in the absence of the compounds. A benznidazole (Bz) control was run in parallel. The activity of the compounds was expressed by the EC_{50} and EC_{90} values.

2.3.5. Anti-T. cruzi activity in vivo

The evaluation of compound **11** on experimental *T. cruzi* infection was performed using male Swiss Webster mice (18−20 g, 4−5 weeks of age) obtained from the animal facilities of the Institute of Science and Biomodels Technology (ICTB) Fiocruz. Mice were housed at a maximum of 5 per cage, kept in a specific-pathogen-free room at 20–24 °C under a 12 h light and 12 h dark cycle, and provided sterilized water and chow ad libitum (Guedes-da-Silva et al., 2015). Animals were acclimated for 7 days before starting the experiments. Infection was performed by intraperitoneal (i.p.) injection of 10^4 bloodstream trypomastigotes (Y strain). *T. cruzi*-infected mice were treated for 5 consecutive days with 0.1 mL of 25 mg/kg/day Bz and 26.65 mg/kg/day compound **11** (dose adjusted to the molecular weight equivalence of 25 mg/kg/day for Bz) by oral gavage or i.p., respectively. The drug administration started at the 5 dpi, which corresponds the parasitemia onset in this experimental model. Only mice with positive parasitemia were used in the infected groups. ALIP19 was diluted in 10 % DMSO +5% Arabic gum and Bz with 3% Tween 80 + H2O in sterile and distillated water. The compound

solutions, freshly prepared before each administration, were dosed according to the body weight of the animals. Each experimental animal group (n = 5) was composed and the parasitemia level was determined individually by direct analysis under a light microscopy, counting the number of parasites in the animal blood (5 μL) collected from the tip of the tails. Mice were weighed once a week to monitor body weight change. Mortality was checked daily up to 30 days after treatment and expressed as percentage of cumulative mortality (Ferreira de Almeida Fiuza et al., 2018). All experimental procedures using animals followed the Biosafety Guidelines in compliance with the Fiocruz regulation, with prior approval by the Committee of Ethics for the Use of Animals (CEUA numbers L038-2017 and L-038/2017-A4).

3. Results

3.1. Compound type selection

A preliminary evaluation of the anti-trypanosomal activity of the test compounds was performed *in vitro* using the intracellular form (amastigote) of *T. cruzi* in L929 mice fibroblastic cell lines. In this case, the cells are infected with amastigote forms of a *T. cruzi* strain (β-galactosidase-transfected Tulahuen strain, DTU VI). A chromogenic β-galactosidase substrate (chlorophenol red-β-D-galactopyranoside, CPRG) is used as a colorimetric reagent. Antiparasitic and cytotoxic effects were evaluated in parallel, to determine the compounds activity (EC_{50}, EC_{90}) and the selectivity index (SI), after 96 h of drug treatment. Benznidazole (Bz), the front-line drug used to treat infections with *T. cruzi*, was used as a control. The results are collated in Table 2.

Out of the six phenyl-pyrazolone derivatives, five compounds were found to be totally inactive, as well as edaravone. These included the phenyl-pyrazolone **1**, the diphenyl-pyrazolones **2** and **3**, the adamantane derivative **4**, and the dimer **5**. In sharp contrast, the pyridine type compound **6** revealed a marked anti-trypanosomal activity, coupled with a moderate cytotoxicity. The level of activity of the compound was close to that measured with the reference product benznidazole, with an EC_{50} value in the low micromolar range. Compound **6** displays a marked activity against *T. cruzi* in L929 cells. The compound is not devoid of cytotoxicity, but the selectivity index (SI = 20) remains satisfactory.

The evaluation was extended using the bloodstream trypomastigote form of *T. cruzi* (Y strain, DTU II). The number and viability of the parasites were determined under light microscopy, after 2 h and 24 h of drug exposure at 37 °C. Here again, the compounds edaravone, **1**, **2**, **3**, **4** and **5** were found to be inactive (EC_{50} > 20 μM), whereas **6** showed a potent activity. After 2 h of incubation with this compound, the calculated EC_{50} and EC_{90} were 0.512 ± 0.66 μM and 0.721 ± 0.52 μM, respectively. After 24 h, EC_{50} and EC_{90} values were 0.441 ± 0.30 μM and 0.930 ± 0.84 μM, respectively. There is no doubt that this pyridine-type compound **6** is a potent anti-trypanosomal agent, with a higher activity than Bz against the bloodstream trypomastigote form of *T. cruzi*.

Based on this preliminary evaluation, a second subset of 8 compounds all bearing a pyrido-pyrazolone scaffold was selected (Fig. 1b). The series includes compound **7** which is the pyridine equivalent of edaravone, and the unfused tricyclic molecules **8**–**14**. They bear one or two methoxy, chlorine or fluorine atoms on the phenyl unit linked to the common pyridyl-pyrazolone scaffold (Fig. 1b).

3.2. Comparison of pyridyl-pyrazolones

The compounds were first evaluated for their cytotoxicity against mammalian cells, after 24 h incubation at 37 °C. No cytotoxicity was observed, be it with rat H9c2 cardiomyocytes or with HepG2 human hepatocellular carcinoma cells. In all cases, the LC_{50} was >300 μM. Then we repeated the viability test with L929 mice fibroblasts but for a longer incubation (96 h). The cytotoxicity of the compounds varies from 20 to 85 μM (Table 3) which is a sign of a moderate cytotoxicity in general. The bicyclic edaravone-like compound **7** is less cytotoxic than the tricyclic molecules. The bis-methoxy compound **8** is twice more cytotoxic than the corresponding bis-chloro compound **9**. There is no highly toxic compound in this series, as expected.

An evaluation of the anti-trypanosomal activity of the compounds was performed using the bloodstream form of *T. cruzi* (Trypomastigotes) after 24 h of incubation. The data are collated in Table 4. The test was performed with the Y strain as described above. Remarkably, we found that several of the compounds, but not all, presented a high activity against the parasite. The two compounds bearing methoxy (**8**) or chlorine (**9**) groups on the phenyl ring were both inactive, as observed with the short bicyclic molecule **7**. In sharp contrast, compound **10** with no substituent on the phenyl ring revealed a remarkable sub-micromolar activity identical to that of the 4′-chloro derivative, whereas the 2′-chloro derivative is a little less active. The most potent compound in the series is **11**, with an outstanding anti-trypanosomal action (EC_{50} = 0.28 μM) superior to that of benznidazole and a remarkable selectivity index (SI > 1000), compared to its effect towards HepG2 and H9C2 cell lines after 24 h of drug exposure (LC_{50} > 300 μM). This compound with a single 4′-methoxy group on the phenyl ring was thus selected as our hit molecule. It showed a clear dose-dependent activity against both parasite forms relevant for mammalian infection (Fig. 2), reaching 100% of the intracellular parasite death at 10 μM after 96 h of incubation (Table 3).

The anti-trypanosomal activity was quantified with the determination of the EC_{50}/EC_{90} ratio for each compound, using the intracellular form of *T. cruzi*, after 96 h of treatment (Table 3). These measurements confirmed that compound **11** was the best in the series, providing EC_{50}/

Table 2
Anti-trypanosomal and cytotoxic activities.

Compounds #	Intracellular *T. cruzi*		L929 cell lines	SI
	EC_{50}	EC_{90}	LC_{50}	
Benznidazole	1.3 ± 0.08	2.99 ± 0.61	>400	>303
Edaravone	>40	ND	>400	ND
1	>40	ND	>400	ND
2	>40	ND	384.9 ± 2.0	ND
3	>40	ND	146.1 ± 37.4	ND
4	>40	ND	>400	ND
5	>40	ND	>400	ND
6	1.6 ± 0.11	4.8 ± 2.52	28.1 ± 3.5	20.7

EC_{50} and EC_{90}: mean ± SD (μM). The intracellular form of *T. cruzi* (Tulahuen strain) was cultured in L929 cells (LC_{50}: mean ± SD -μM) for 96 h at 37 °C. SI, Selectivity Index. Effective concentration for 50% or 90% inhibition (EC_{50}, EC_{90}). Median (50%) lethal concentration (LC_{50}).

Table 3
Cytotoxicity against L929 mice fibroblasts and activity of the compounds against the intracellular form of *T. cruzi*.

Compounds	L929 cytotoxicity LC_{50} (μM)	Intracell. *T. cruzi* EC_{50} (μM)	Intracell. *T. cruzi* EC_{90} (μM)	SI (LC_{50}/EC_{50})
Benznidazole	>300	1.29 ± 0.26	2.71 ± 0.17	>233
6	28.07 ± 3.48	1.60 ± 0.11	4.80 ± 2.52	17.5
7	85.70 ± 14.08	8.51 ± 3.96	29.70 ± 2.00	10
8	21.16 ± 1.35	>10	>10	ND
9	48.09 ± 19.81	>10	>10	ND
10	28.86 ± 3.21	2.47 ± 0.35	3.16 ± 0.47	12
11	28.22 ± 1.11	1.30 ± 0.30	2.71 ± 0.65	21.7
12	45.42 ± 6.71	5.38 ± 2.69	23.64 ± 4.86	8.5
13	37.11 ± 3.90	3.08 ± 0.10	4.17 ± 0.38	12
14	20.62 ± 1.06	>10	>10	ND

LC_{50}: mean ± SD (μM), measured after 96 h at 37 °C. L929 cells seeded in 96-wells microplates (4 × 10^3 cells/well, in RPMI 1640 medium supplemented with 10 % FBS) in the presence of increasing concentrations of the test compounds. The cellular viability was evaluated using the AlamarBlue assay. SI, Selectivity Index.

Table 4
Activity of the compounds against the bloodstream form of *T. cruzi* (Y strain).

Compounds	EC_{50}[a] (μM) - mean ± SD	SI
Benznidazole	6.7 ± 5	>47
6	0.44 ± 0.30	ND
7	>10	ND
8	>10	ND
9	>10	>ND
10	0.83 ± 0.32	>361
11	0.28 ± 0.19	>1071
12	0.82 ± 0.46	>366
13	1.27 ± 0.64	>236
14	1.37 ± 0.70	>219

[a] EC_{50} were determined with the bloodstream trypomastigote forms of *T. cruzi* (Y strain, DTU II) after 24 h of incubation at 37 °C. Parasites (10^7/mL) were seeded in 96-well microtiter plates in the presence of the test compound, in RPMI medium (containing 5% FBS). The number of live parasites was determined by microscopy using the PrestoBlue assay.

EC_{90} values as good as those determined with the reference Bz (Table 3). Compound **11** was a little better than **6**, suggesting that a single methoxy group on the phenyl ring, at the 3′ or 4' position, is sufficient to contribute favourably to the activity. The two molecules display a similar level of selectivity (SI = 17–21), which is less pronounced that of Bz (SI > 230) but still acceptable. The other compounds in the series were a little less active against the parasite and with a less favorable selectivity index.

3.3. *In vivo evaluation*

Compound **11** is relatively well soluble in aqueous media (measured solubility 199.2 μM) and it presents an acceptable lipophilicity (logD = 2.820). Compound **6** is a little bit less soluble (80.9 μM) and equally lipophilic (logD = 2.565). Unfortunately, both compounds were found to be unstable in mouse CD-1 plasma ($t_{1/2}$ = 12 and 10 min, for **11** and **6**, respectively). The lack of stability in mouse plasma ($t_{1/2}$ < 30 min) is a general problem with all prepared pyrazolones (Le Biannic et al., 2022; de Araújo et al., 2020a). We know that the plasma stability of these molecules must be improved in future drug design. Nevertheless, we attempted to evaluate the anti-trypanosomal activity of best compound in a murine model as a preliminary proof of concept.

The activity of compound **11** was evaluated in male mice infected with bloodstream form of *T. cruzi* (Y strain). Mice were treated on 5th day-post-infection (dpi) for 5 consecutive days with the compound at 26.65 mg/kg/day under intraperitoneal (ip) injection, or with Bz at an equimolar dose of 25 mg/kg. At 5 dpi, the infection is already well established, and parasites are disseminated into different tissues and organs, offering thus a robust model for drug testing. The blood parasitemia was evaluated, together with the variation of weight of the animals (Fig. 3). We observed that Bz reduced the number of blood parasites, whereas **11** showed only a modest effect. The parasitemia peak was reduced by about 40 % with **11** on day 8 but the global evolution of the parasitemia was similar for the control (vehicle-treated mice) and **11** groups. This is reflected also with the weight curves which were almost identical for the control infected and vehicle-treated group and the group receiving **11**, whereas mice receiving Bz showed a favorable weight evolution and a major (90 %) decline of the parasite load. The survival curves confirmed the very modest activity of **11** which only slightly delayed the mortality of mice infected with *T. cruzi*, with a 3-days shift (from d16 to d19) of the 50% mortality level, whereas all mice treated with Bz survived (100% even at d45) (Fig. 3).

4. Discussion

This work has identified two pyridine-pyrazolone derivatives potently active against *T. cruzi in vitro*. Two parasite forms were evaluated, the bloodstream trypomastigotes and intracellular amastigotes. These two distinct DTU (discrete typing units II and VI) are clinically relevant, as they are associated with chronic infections causing cardiomyopathy and digestive megasyndromes in patients (Chatelain and Ioset, 2018). Compounds **6** and **11** display a robust activity against the intracellular form of *T. cruzi*, being equally active as the reference drug benznidazole. The measured IC_{50} values are identical for **11** and benznidazole when using the intracellular form of the parasite (Table 3). In contrast to other pyrazolone series that were found to highly potent against intracellular forms but inactive on bloodstream forms (Sijm et al., 2019, de Araújo et al., 2020a), compound **11** proved to be even 24-time more potent than benznidazole against trypomastigotes and it presents a remarkable selectivity index (SI > 1000) (Table 3). Moreover, the compound did not reveal toxic effect (no increased mortality has been observed). This is interesting because the failure of azole drug candidates, identified as potent *T. cruzi* CYP51 inhibitors in the clinical trials for CD has been correlated in part to their low *in vitro* activity against the non-replicative form of *T. cruzi* (trypomastigotes and quiescent forms) (Chatelain and Ioset, 2018). There is a strong need for novel drugs which could target both the intracellular replicative amastigote and the non-replicative trypomastigote forms. In this context, our *in vitro* data advocate for the development of compound **11** and analogues.

The observations reported here with the pyridyl-pyrazolones open interesting perspectives for the design of anti-trypanosomal agents. Compound **11** displays the typical *in vitro* profile expected for a novel anti-*T. cruzi* drug candidate (EC_{50} < 5 μM, max activity >90%, selectivity >10) (Soeiro, 2022; Chatelain and Ioset, 2018). Unfortunately, this compound revealed only a minor activity *in vivo*, far inferior to that of Bz when tested in an equimolar dose (25 mg/kg), most likely due to the instability of the compound in murine plasma. This is a known limitation in the pyrazolone series (Le Biannic et al., 2022; de Araújo et al., 2020a), but there are options to reduce the chemical instability of the molecule, such as the substitution of the phenyl ring with halogens. The pyrazolone can be modified, while preserving the high affinity of

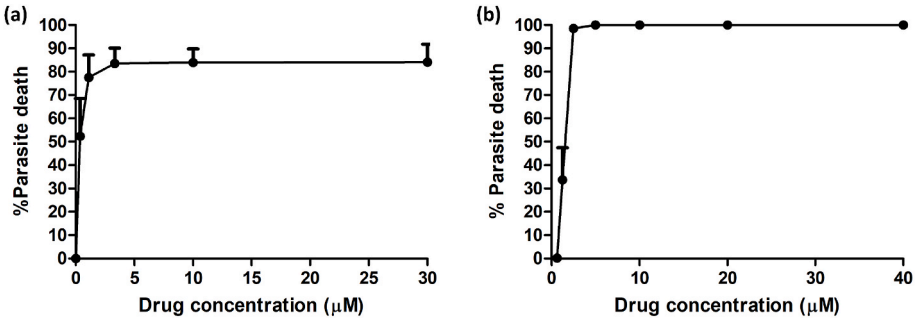


Fig. 2. Dose-dependent activity of compound **11**. (a) Activity against bloodstream trypomastigotes of *T. cruzi* (Y strain) after 24 h of incubation at 37 °C. (b) Activity against intracellular amastigotes of *T. cruzi* (Tulahuen strain) after 96 h of incubation at 37 °C.

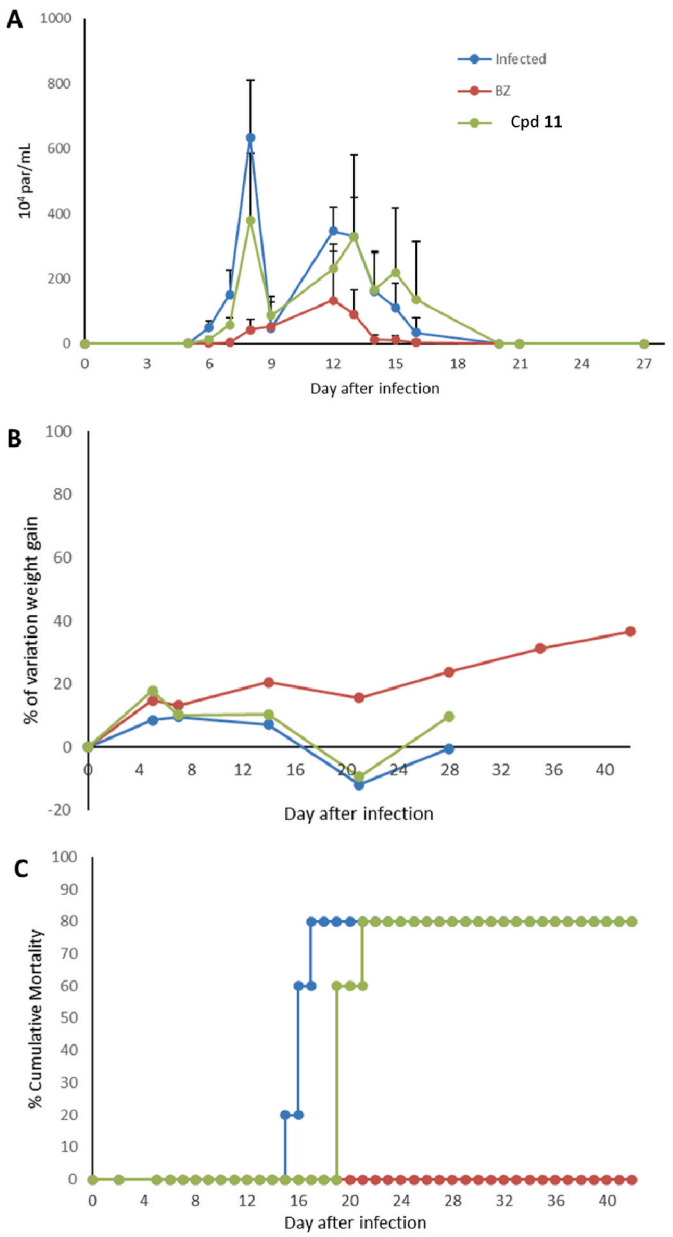


Fig. 3. Parasitemia (A), animal weight (B) and mortality (C) curves in mice infected by *T. cruzi* under compound **11** or benznidazole therapies. In these experiments, Swiss male mice (n = 5) were infected with the bloodstream form of *T. cruzi* (10^4 cells, Y strain) prior to the ip treatment (at 5dpi) with compound **11** or benznidazole for 5 consecutive days.

the molecule for PD-L1. The chemical strategy cannot be disclosed at present for patent priority reason, but we can refer to similar approaches used to reduce the metabolic instability of molecules with a high electrophilic group for example (Li et al., 2021; Zhu et al., 2022). Novel pyrazolone- and pyrazole-bearing compounds potently targeting PD-L1, stable in murine plasma, will be reported in due course (patent application under review).

Compound **11** is a potent PD-L1 binder. Its high affinity for recombinant human PD-L1 measured by MST (K_D = 83 nM) has been confirmed by another method, isothermal titration calorimetry (ITC; K_D = 120 nM). The compound efficiently blocked the PD-1/PD-L1 interaction in cells (IC_{50} = 17 nM) and reactivated the proliferation of dormant CTLL-2 cells in the presence of hPD-L1 (IC_{50} = 75 nM) (data not shown). But here, the mechanism of action of the active compounds **6** and **11** is not PD-1/PD-L1 checkpoint-dependent under our experimental condition *in vitro*. There is no PD-1 expressing immune cells in the assay. In addition, most of the pyridine-pyrazolone derivatives tested here are good inhibitors of PD-L1 (Table 1) but only a few of them display an activity against *T. cruzi*. Moreover, there no direct correlation with the antioxidant capacity of the compounds because, for example compound **2** was previously shown to be a potent scavenger for oxygen free radicals (Regnault et al., 2023) but it is inactive here against the parasite. The molecular origin of the anti-*T. cruzi* activity of compound **11** remains unknown at present. Nevertheless, the compound and the series are interesting, considering also that these compounds are readily accessible by chemical synthesis. Low-cost products are absolutely required for use in low-income countries where the parasite is endemic.

The situation is reminiscent to that reported with other pyrazolones active against the same parasite, with potent molecules but no target identified at present (Sijm et al., 2019, 2020, 2021). A hypothesis can be proposed. There is the possibility that these compounds function as inhibitors of phosphodiesterases (PDEs) to increase the cellular concentration of cyclic AMP (cAMP) which plays a vital role in *T. cruzi* metabolism. This has been reported with the phenyl-pyrazolone derivative NPD-227, which is a potent anti-trypanosomal pyrazolone characterized recently (de Araújo et al., 2020a). Different anti-trypanosomal molecules capable of elevating intracellular cAMP content in both bloodstream trypomastigotes and amastigotes have been discovered (de Araújo et al., 2019, 2020b). There are different trypanosomatid phosphodiesterases considered as potential drug targets, such as TcrPDE-A1, involved in the epimastigotes response to oxidative stress (Sternlieb et al., 2020). There are pyrazolone-type inhibitors of PDE active against *Trypanosoma* (Amata et al., 2015) including compound shown to increase intracellular cAMP levels in *T. brucei* and *T. cruzi* trypanosomes (de Araújo et al., 2019, 2020b; Orrling et al., 2012). It will be interesting to investigate the effect of our compounds on cAMP levels in extracellular amastigote and bloodstream forms of *T. cruzi*. A preliminary molecular docking analysis with compound **11** bound to the catalytic domain of *T. cruzi* phosphodiesterase C1 (TcrPDEC1, PDB access code 3V94) (Wang et al., 2012) strongly suggests that this compound can form stable complexes with the enzyme (Fig. 4). The calculated empirical energy of interaction (ΔE) was −54.05 kcal/mol for compound **11**, even better (more negative) than to the value calculated with the crystallographic ligand wyq16 (ΔE = −51.60 kcal/mol) under identical conditions. There is a large binding pocket (with a volume of 1157 $Å^3$) suitable to accommodate the pyridyl-pyrazolone ligand. Remarkably, the compound engages two H-bonds with residue Tyr367 of the enzyme and two p-stacking interactions between the phenyl unit of **11** and protein residues Phe573 and Phe577 stabilize the complex (Fig. 4c). The molecular modeling analysis is very informative, suggesting that *T. cruzi* phosphodiesterase may represent a privileged binding target for 19, possibly at the origin of the anti-trypanosomal activity. This hypothesis will be investigated further.

In conclusion, we have identified a pyridyl-pyrazolone derivative potently active against *T. cruzi in vitro*. The level of activity of compound **11** is similar to that of the commercial drug benznidazole. A minor activity was observed in a murine model of mice infected with *T. cruzi*, probably because of the very limited plasma stability of the molecule. Nevertheless, the compound is easy to synthesize and can be modulated to reinforce its stability. Preliminary structure-activity information is given to guide future lead optimization. The study opens novel perspectives for the possible design of safe and effective anti-trypanosomal agents. The mechanism behind the activity of compound **11** warrants further investigation.

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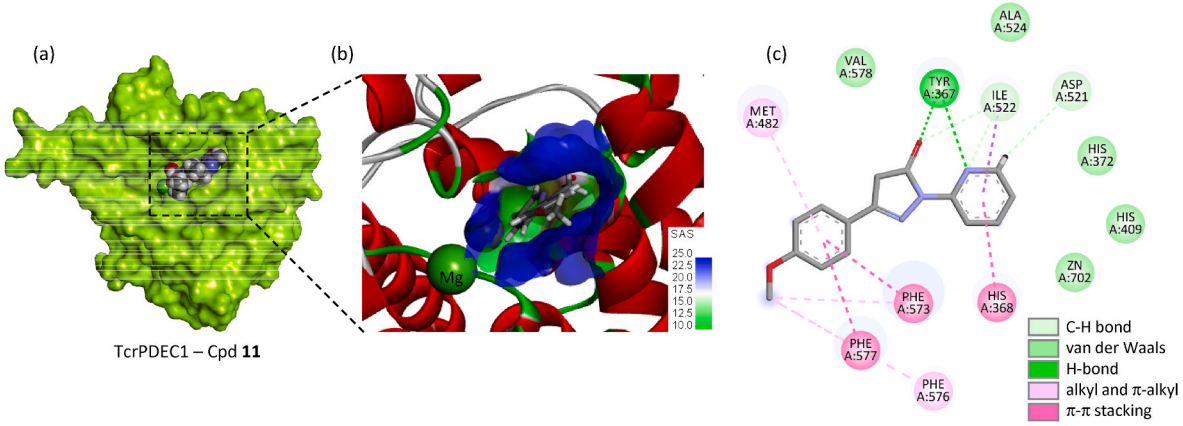

Fig. 4. Molecular model of compound **11** bound to of *T. cruzi* phosphodiesterase C1 (3V94). (a) A surface of compound **11** bound to TcrPDEC1. (b) A close-up view of the compound inserted into the binding cavity, with the solvent-accessible surface (SAS) surrounding the drug binding zone (color code indicated). (c) Binding map contact for compound **11** bound to TcrPDEC1 (color code indicated). The modeling analysis was performed according to a published procedure (Thuru et al., 2023).

and O.M are CNPq and CNE fellows.

CRediT authorship contribution statement

Denise da Gama Jaen Batista: Investigation, Formal analysis, Data curation. **Ludmila Ferreira de Almeida Fiuza:** Investigation, Formal analysis, Data curation. **Frédérique Klupsch:** Investigation, Formal analysis. **Krislayne Nunes da Costa:** Methodology, Investigation, Formal analysis, Data curation. **Marcos Meuser Batista:** Methodology, Formal analysis, Data curation. **Ketlym da Conceição:** Investigation, Formal analysis, Data curation. **Hassiba Bouafia:** Investigation, Formal analysis. **Gérard Vergoten:** Software. **Régis Millet:** Supervision. **Xavier Thuru:** Supervision, Formal analysis. **Christian Bailly:** Writing – review & editing, Writing – original draft, Validation, Supervision, Conceptualization. **Maria de Nazaré Correia Soeiro:** Writing – review & editing, Writing – original draft, Validation, Supervision, Investigation, Data curation, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Data availability

Data will be made available on request.

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