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Resistance

7-Aryl-7-deazapurine 3′-deoxyribonucleoside derivative as a novel lead for Chagas' disease therapy: *in vitro* and *in vivo* pharmacology

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Background: The protozoan *Trypanosoma cruzi* is auxotrophic for purines and causes Chagas' disease (CD), a neglected illness affecting >6 million people. Combining the 3-deoxyribofuranose part of cordycepin with the modified purine ring of a nucleoside 'hit' led to the discovery of 4-amino-5-(4-chlorophenyl)-N7-(3′-deoxy-β-D-ribofuranosyl)-pyrrolo[2,3-d]pyrimidine (Cpd**1**), revealing promising anti-*T. cruzi* activity.

Objectives: To further evaluate Cpd**1** *in vitro* and *in vivo* to fully assess its therapeutic potential against CD, covering cell culture sterilization through washout assays, drug combination with benznidazole and long-term administration in *T. cruzi*-infected mice.

Results: Although less susceptible to Cpd**1** than amastigotes, trypomastigotes present an impaired capacity to successfully establish intracellular infection of cardiac cultures. Combination of benznidazole with Cpd**1** indicated no interaction (additive effect) (FIC index = 0.72) while administration to mice at one-tenth of the optimal dose (2.5 mg/kg and 10 mg/kg for Cpd**1** and benznidazole, respectively) suppressed parasitaemia but failed to avoid mortality. Long-term treatment (60 days) gave a rapid drop of the parasitaemia (>98% decline) and 100% mice survival but only 16% cure. *In vitro* washout experiments demonstrated that although parasite release into the supernatant of infected cardiac cultures was reduced by >94%, parasite recrudescence did occur after treatment.

Conclusions: Parasite recrudescence did occur after treatment corroborating the hypothesis of therapeutic failure due to subpopulations of dormant forms and/or genetic factors in persister parasites involved in natural drug resistance.

Introduction

American trypanosomiasis, known as Chagas' disease (CD), was discovered in 1909 by Carlos Chagas and is largely ignored in terms of R&D investment.[1] CD is caused by the protozoan *Trypanosoma cruzi* and has two clinical disease stages. The asymptomatic or oligosymptomatic acute phase lasts up to 2 months and is characterized by patent parasitaemia. Due to an efficient host immune response, the parasitism is controlled but not eliminated and the disease moves to the chronic stage in which most infected people will present an asymptomatic profile. After years or even decades for reasons not fully known yet, 30%–40% develop progressive cardiac and/or digestive complications with fatal outcome.[2–4] The nature of the host–parasite interaction as well as the host's and parasite's genetic backgrounds may play a role in these symptomatic manifestations.[5] Also, comorbidity with the new coronavirus SARS-CoV-2 is a threat for chagasic patients.[6]

Substantial improvement in vector control was achieved in several endemic countries, and the number of new cases dropped considerably. However, it still poses a public concern due to remaining/new triatome foci,[7] besides congenital and oral transmissions.[8]

Only two old nitro-derivative drugs are available: benznidazole and nifurtimox.[9] Both have low efficacy in the chronic stage, limiting a therapeutic benefit for millions of infected individuals.[10,11] Also, they require long-term administration and elicit several side effects leading to low patient adherence or discontinuation of treatment. Finally, the nitro-drugs are contraindicated in pregnant women and in people suffering from liver or kidney failure or neurological disorders.[9,12,13] Novel, safer and more efficacious drugs are therefore mandatory.[14]

Like other trypanosomatid protozoa, *T. cruzi* depends on uptake of host purines as it is not able to synthetize purine rings *de novo*, rendering interference with the purine salvage attractive.[15,16] In fact, purine nucleoside analogues have a wide spectrum of activity, including antiviral,[17] antitumoral[18] and antibacterial.[19] Some nucleoside derivatives exhibit nano- and submicromolar *in vitro* activity against African trypanosomes,[20] *Leishmania* spp.[21] and *T. cruzi*.[22,23]

After revisiting the anti-trypanosomatid activity of the natural nucleoside antibiotic tubercidin and a series of C-7 modified analogues, phenyl-substituted compounds showed encouraging efficacy against intracellular forms of *T. cruzi,* being more potent than benznidazole, which motivated us to combine 7-substituted 7-deazapurine moieties with the carbohydrate group of cordycepin.[22,23] After rounds of optimization, compound **1** (Cpd**1**) or 4-amino-5-(4-chlorophenyl)-N7-(3′-deoxy-β-ᴅ-ribofuranosyl)-pyrrolo[2,3-d]pyrimidine (Figure 1) revealed outstanding efficacy in an acute mouse model of CD, however, without resulting in parasitological cure.[23]

In the present study, additional *in vitro* and *in vivo* protocols were performed to assess and improve the efficacy of Cpd**1**, including drug combination approaches with benznidazole, washout assays, pretreatment of trypomastigotes prior to incubation with host cells, and dose-titration and long-term (60 days) drug administration experiments in mice aiming to control parasite relapses.

Materials and methods

Compounds

Benznidazole (LAFEPE, Brazil) was used as reference drug. Cpd**1** (Figure 1) was synthesized as described by Hulpia *et al.*[23] The identity and purity of Cpd**1** was confirmed via ^{1}H NMR, ^{13}C, HRMS and analytical LC/MS respectively. The analytical data match those previously reported.[23] Purity of Cpd**1** was >95%. Stock solutions of benznidazole and Cpd**1** were prepared in 100% DMSO, with the final in-test concentration never exceeding 0.6% for *in vitro* experiments to avoid non-specific toxicity.[24]

Mammalian cell culture

Primary cultures of mouse embryonic cardiac cells (CC) were obtained as described.[25,26] L929 cell lines were cultivated (4.000 cells/well) in 96-well microplates at 37 °C in RPMI 1640 medium (Sigma–Aldrich).[27]

Parasites

Bloodstream trypomastigotes (BT) of Y strain (DTU II) were obtained as reported.[24] The trypomastigote forms of Tulahuen strain (DTU VI) expressing the *Escherichia coli* β-galactosidase gene were collected from the supernatant of L929 cell cultures previously infected (host: parasite cell ratio 10:1).[27] For both strains, purified parasites were added to RPMI 1640 medium supplemented with 5% FBS to perform assays at 37 °C in 5% CO_2.

In vitro assays

The infections were performed using parasite: host cell ratio 10:1, at least two assays in biological duplicate. Half inhibitory concentration (EC_{50}) and statistical analysis (95% CI of SD) were obtained through non-linear regression analysis by GraphPad Prism Version 9.1.0.

Pretreated BT forms

Bloodstream trypomastigotes were incubated for 24 h with Cpd**1** and benznidazole at their corresponding EC_{50} (23 ± 4 and 11.5 ± 1.1 µM, respectively).[23] Parasites were rinsed to remove compound, and the number of live and motile parasites determined by light microscopic quantification to adjust the parasite: host cell ratio. After 24 h of interaction, the infected CC were rinsed to get rid of non-internalized parasites, fixed with Bouin or submitted to additional incubation up to 72 h.[28] After fixation and staining with Giemsa, light microscopic analysis was performed to determine the number of infected host cells, parasites per infected cell and infection index (percentage of infected cells multiplied by the average number of intracellular parasites per infected host cell).

Washout assays

After 24 h of plating, CC were infected for 24 h at 37 °C with BT (Y strain). The cultures were rinsed to remove free parasites and treated for 168 h at 37 °C with the compounds at the non-toxic concentration of 5 µM in culture medium that was replaced every 48 h. After 168 h of drug exposure, the cultures were rinsed using PBS and drug-free culture medium was added for another 168 h of incubation. Parasites released into the medium were quantified using light microscopy.[22]
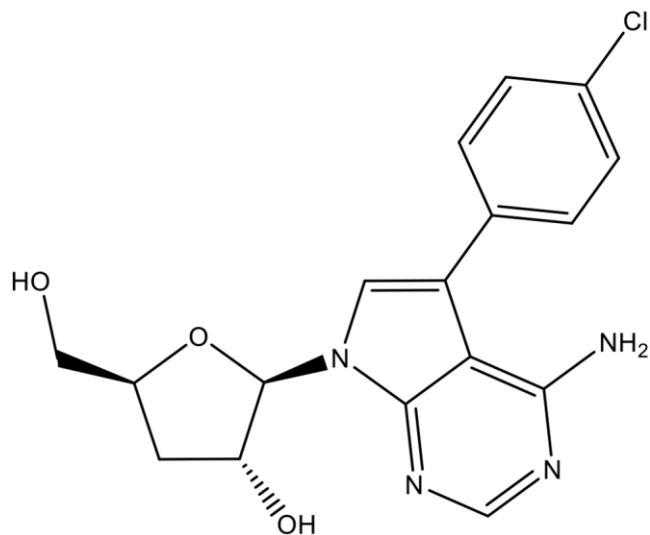
Figure 1. Chemical structure of Cpd**1**: 4-amino-5-(4-chlorophenyl)-N7-(3′-deoxy-β-ᴅ-ribofuranosyl)-pyrrolo[2,3-d]pyrimidine.

Drug combination assays

The association of benznidazole + Cpd**1** was assessed by colorimetric read-out using L929 cells that were infected for 2 h with trypomastigote forms (Tulahuen-β-gal strain), followed by rinsing the cultures to remove non-internalized parasites and further incubated for 24 h before drug administration using a fixed-ratio method.[29,30] Predetermined EC_{50} values were used to determine the top concentrations of each drug ensuring that EC_{50} fell at 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.[30,31] At least two independent experiments in triplicate were performed. The FIC index (FICI) at the EC_{50} of each drug was calculated as EC_{50} when in combination/EC_{50} of drug alone. The sum of FICI of each compound (ΣFICI) was obtained.[31] The mean ΣFICI (xΣFICI) was calculated as the average of ΣFICI. Isobolograms were built by plotting the FICI values. The ΣFICI was used to classify the nature of interaction as recommended by Odds:[30] synergy for xΣFICI ≤0.5, no interaction for xΣFICI 0.5–4 and antagonism for xΣFICI >4.

In vivo activity

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 were housed at a maximum of six per cage, kept in a specific-pathogen-free room at 20 °C to 24 °C under a 12 h light and 12 h darkness cycle, and provided sterilized water and chow *ad libitum*. The animals were acclimated for 7 days before starting the experiments. Infection was performed by intraperitoneal (IP) injection of 10^4 bloodstream trypomastigotes (BT) (Y strain). The animals were divided into the following groups ($n \geq 5$): untreated (infected but treated only with citrate buffer vehicle) and treated (infected and treated with the compounds). *T. cruzi* (Y strain)-infected mice were treated by oral gavage for 60 consecutive days (except for weekends) starting at 5 days post-infection (dpi), which corresponds to the parasitaemia onset, using 0.25–25 mg/kg (q12h) of Cpd**1** or 100 mg/kg/day benznidazole. Co-administration for 11 days at suboptimal doses of Cpd**1** (2.5 mg/kg/day) + benznidazole (10 mg/kg/day) was also performed. Benznidazole at 100 mg/kg/day as the optimal dose was run in parallel. Cpd**1** was diluted in 10% (v/v) EtOH, 0.1 M aqueous citrate buffer (pH 3.02, 1.8 mg/mL and then dosed according to body weight of the animals and drug concentration). The drug formulations were freshly prepared before each administration. Only mice with positive parasitaemia were used in the infected groups. Parasitaemia was individually checked by direct microscopic counting of parasites in 5 μL blood, and mice were examined daily for mortality until 261 dpi when blood was collected from surviving animals for qPCR analysis. Animal survival is expressed as the percentage survival rate at the endpoint. For blood qPCR analysis, 500 μL of blood was diluted in a 1:2 volume of guanidine solution and heated for 60 s in boiling water, followed by DNA purification using a High Pure PCR Template Preparation Kit (Roche Applied Science) and quantitative Realtime multiplex PCR assays. TaqMan probes were used for quantification of both *T. cruzi* satellite nuclear DNA and internal amplification control (IAC) (Figure S1, available as Supplementary data at *JAC-AMR* Online).[32] Standard curves were constructed for absolute quantification through the serial dilution of total DNA, ranging from 10^5 parasite equivalents to 1 parasite equivalents per mL of blood, obtained with a negative blood sample in guanidine-EDTA. Parasite load was then expressed as equivalent of parasite DNA per mL of blood.[33] All animal studies were carried out in strict accordance with the guidelines established by the FIOCRUZ Committee of Ethics for the Use of Animals (CEUA L038-2017).

Statistical analysis

Statistical analysis was performed using ANOVA with the level of significance set at $P \leq 0.05$.

Results

Pretreatment of BT

BT were incubated with EC_{50} of Cpd**1** or benznidazole. Drug-treated and untreated parasites were rinsed, and live and motile trypomastigotes quantified before infection of CC. After 24 h, infected cultures were rinsed to remove non-internalized trypomastigotes and followed-up for up to 72 h (Figure 2). Although not presenting high potency, Cpd**1** induced morphological alterations (round-shape like phenotype) suggestive of impaired fitness. When treated parasites were used to infect CC, although no major effect could be found after 24 h post-infection (hpi), a high and statistically significant ($P < 0.025$) drop in all parameters (percentage of infected host cells, parasites per infected cell and infection index rates) was observed for Cpd**1** pretreated trypomastigotes after 48 and 72 h of CC infection. With benznidazole pretreated parasites, the mean parasites per infected cell values gave a statistically relevant decrease ($P = 0.0135$) only at 48 hpi, while after 72 h all parameters were reduced ($P \leq 0.0093$) (Figure 2a–d). Cpd**1** and benznidazole interfere with parasite fitness, impairing the parasite's ability to successfully establish and sustain infection *in vitro*, affecting the proliferative capacity as clearly shown at 72 hpi with earlier and greater reduction rates triggered by Cpd**1** leading to >90% decrease in the infection indexes (Figure 2c).

Drug combination

In vitro effect of co-treatment of Cpd**1**[23] and benznidazole was assessed by colorimetric readout, using L929 cells infected with β-gal Tulahuen strain with a fixed-ratio method.[29–31] The result of xΣFICI equal to 0.72 obtained from Cpd**1** and benznidazole combination confers the status of a merely additive effect (Figure 3). The most promising results were obtained with the drug ratio of 1:4 (Cpd**1**: benznidazole) leading to the lowest IC_{50} and ΣFICI = 0.27 revealing a synergistic profile (Figure 3a). The isobolograms (Figure 3b) show the drug interaction profile with almost all ratios below the graphic threshold, except for 4:1 Cpd**1**: benznidazole ratio (ΣFICI = 1.11).

A proof-of-concept *in vivo* co-administration study was conducted with Cpd**1** and benznidazole in male Swiss mice infected with Y strain (five per group).[27] The chosen drug ratio was near to that which gave the best results *in vitro* (synergy profile reached at 1:4 of Cpd**1**: benznidazole ratio). The treatment started on the onset of parasitaemia (5 dpi) and was given orally for 11 days q12h for Cpd**1** and q24h for benznidazole. Mice also received each drug alone (Cpd**1** at 2.5 mg/kg and benznidazole 10 mg/kg), benznidazole at its optimal dose (100 mg/kg) or just vehicle.

Cpd**1**, benznidazole and their combination gave similar declines in parasite load, reaching ≥94% at peak on 8 dpi (Figure 3c). Cpd**1** (2.5 mg/kg) given alone resulted in 60% of animal survival. However, mice treated with benznidazole + Cpd**1** showed increased death rates (80% mortality) and enhanced toxicity, exhibiting urination impairment, prostatic and hunched postures with weight loss. All surviving mice in the different groups showed parasite relapse after interrupting drug administration. Based on the still promising results of Cpd**1** in monotherapy at 2.5 mg/kg producing >97% reduction in blood parasitaemia, follow-up experiments were conducted adopting dose-dependent analysis with reduced Cpd**1** doses and introducing long-term drug
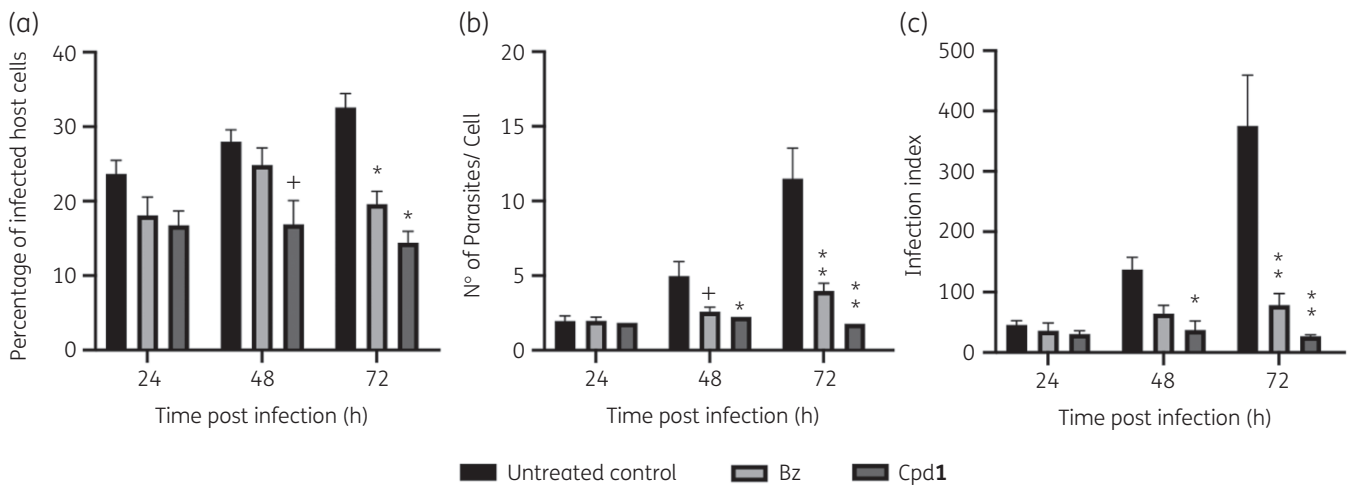
Figure 2. Pretreatment assays of *T. cruzi* (Y strain) with Cpd**1** or benznidazole (Bz) before parasite–host cell interaction. BT were treated or not (untreated control) for 24 h with the compounds (corresponding EC_{50}), rinsed and used to infect cardiac cell cultures (moi 10:1) in drug-free medium. The mean and standard deviation values were plotted according to percentage of infected host cells (a), number of parasites/cell (b) and infection index (c), at each timepoint. Statistical analysis was performed by two-way multiple comparison ANOVA Tukey's test ($P < 0.05$, 95% CI). $^{+}P < 0.05$, *$P < 0.01$ and **$P < 0.001$.

(a)

Drug ratio	EC_{50} (µM) Mean±SD		FICI		ΣFICI	xΣFICI
	Cpd1	Bz	Cpd1	Bz		
5:0	0.246±0.097					
4:1	0.101±0.064	1.396±0.097	0.41	0.70	1.11	
3:2	0.082±0.059	1.059±0.064	0.33	0.53	0.86	
2:3	0.056±0.045	0.695±0.059	0.23	0.35	0.57	0.72
1:4	0.028±0.024	0.319±0.045	0.11	0.16	0.27	
0:5		2.006±1.024				

(b)

(c)
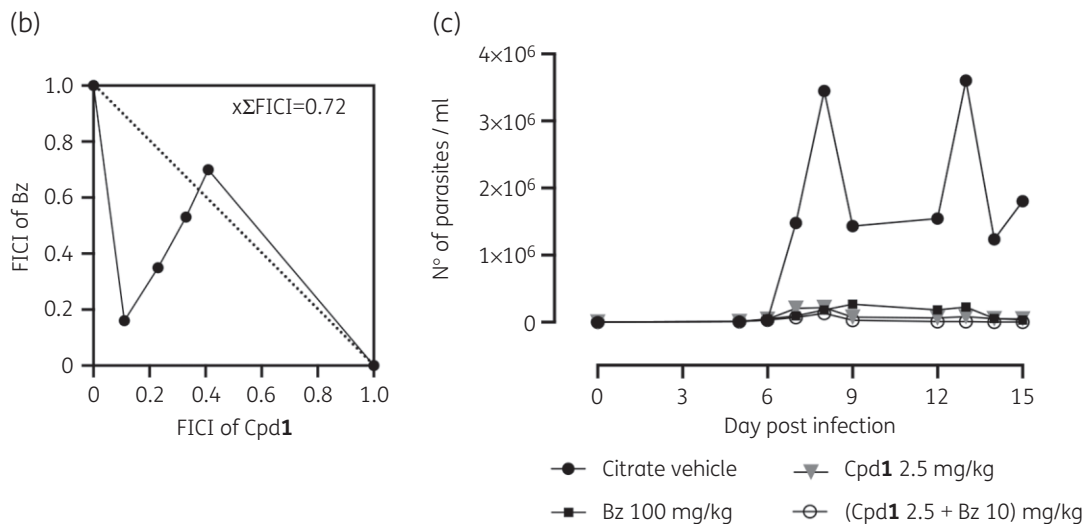
Figure 3. *In vitro* (a and b) and *in vivo* (c) analysis of Cpd**1** and benznidazole (Bz) combination. Isobolograms (b) of FICI values (a) were calculated based on the *in vitro* activity (EC_{50}) against intracellular forms of *T. cruzi* after 168 h of drug exposure. (c) *In vivo* evaluation of Cpd**1** in *T. cruzi* mouse model of acute infection displaying parasitaemia levels of animal groups. Male Swiss mice were infected (IP) with 10^4 BT (Y strain) and drug administration (11 days) started at 5 dpi. Benznidazole alone was given at 100 mg/kg (q24h, orally) as a reference drug given at the optimal dose. Co-administration of benznidazole (10 mg/kg, q24h, orally) and Cpd**1** (2.5 mg/kg, q12h) is depicted (c). Control group received only vehicle (citrate buffer, q12h).
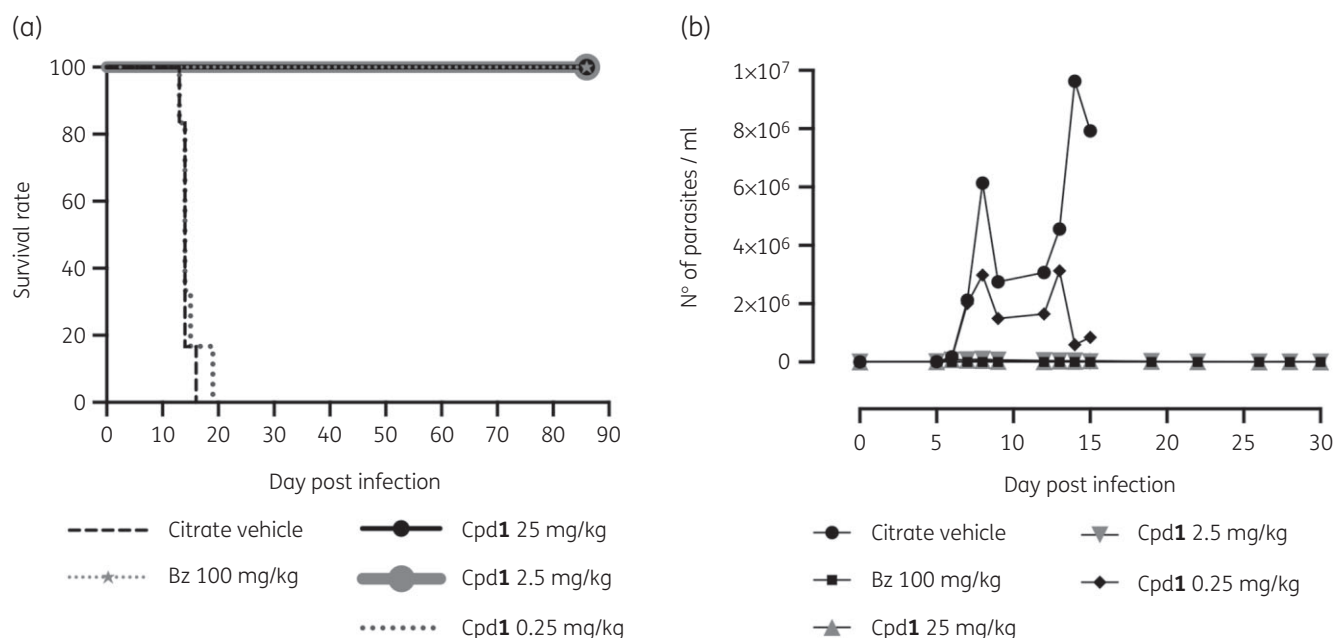
Figure 4. *In vivo* evaluation of Cpd**1** in a mouse model of *T. cruzi* acute infection. (a) Survival rates (b) and parasitaemia levels of animal groups. Male Swiss mice were infected (IP) with 10^4 BT (Y strain). Cpd**1** administration (0.25–25 mg/kg, q12h, orally) was initiated at 5 dpi and given for 60 days. Benznidazole (Bz) was included as a reference drug (100 mg/kg, once a day, orally). Control group received only vehicle (citrate buffer, q12h).

administration (60 days) aiming to check whether sterile cure could be achieved.

### In vivo dose-titration and long-term drug administration

To check for parasite sterilization, an extended and dose-response protocol of drug administration (Cpd**1** at 0.25–25 mg/kg for 60 days, excluding weekends) was used in the same mouse model of acute *T. cruzi* infection. Cpd**1** showed a dose-dependent decrease in both parasitaemia levels and mortality rates (Figure 4a and b). At 25 and 2.5 mg/kg, a sharp drop (>98%) in blood parasite load was reached similarly to benznidazole at 100 mg/kg (Figure 4a). Cpd**1** at 0.25 mg/kg resulted in >50% of parasitaemia decline although was not able to prevent animal mortality (Figure 4a and b). Blood analysis by qPCR of the surviving animals[33] showed that the standard curves ranged from 10^5 to 1 parasite equivalents per mL of blood. Parasite load referred as equivalent of parasite DNA per mL of blood sample (parasite equivalents/mL) showed 76% efficiency for the satellite nuclear DNA target with a linearity coefficient of 0.996 (Figure S1b), confirming the sensitivity and accuracy of parasite detection and quantification. When infected mice were treated with benznidazole, three out of five animals presented negative blood qPCR (60% of cure) while another showed very low amplification (0.0247 parasite equivalents/mL) (Figure 5). Doses of 25 and 2.5 mg/kg of Cpd**1** also decreased the blood parasite load but to a lesser extent than benznidazole, with the one animal from the 25 mg/kg group displaying negative qPCR (17% cure) (Figure 5). Statistical analysis indicated no significant differences between the groups (ANOVA $P > 0.31$).

Washout assays

The number of released parasites into the supernatant of the infected CC cultures was quantified by light microscopy at different timepoints (Figure 6a).

Both benznidazole and Cpd**1**-treated cultures sustained a marked and significant ($P \leq 0.001$) reduction in the number of released parasites compared with the untreated samples, which showed a continuous discharge of trypomastigotes into the culture medium peaking at 144 h with >200 parasites/mL (Figure 6a and b). Before drug withdrawal, Cpd**1** fully suppressed parasitism (light microscopy observation) while benznidazole resulted in a minor parasite release (≈ 8 parasites/mL) at 144 hpi (Figure 6a). After medium washout at 168 h, benznidazole and Cpd**1** were able to sustain the absence of parasites into the culture supernatant for 24 h, while untreated cultures exhibited >75 parasites/mL. However, trypomastigotes were identified from the fourth day of drug removal (at 264 h) in benznidazole as well as Cpd**1**-treated cultures. Benznidazole and Cpd**1** incubation resulted in comparable parasitism (6 and 10 parasites/mL, respectively) that was at least 19-fold lower than the untreated group (180 parasites/mL). At the last timepoint (312 h or sixth day post-drug withdrawal), the number of released parasites reached 15 (Cpd**1**) and 13 (benznidazole) parasites/mL, being about 15-fold lower than the untreated control (>210 parasites/mL). Washout assays demonstrated a similar capacity of Cpd**1** and benznidazole to temporarily arrest parasite growth, ensuing a reduced number of released parasites into the supernatant of infected CC cultures. Cpd**1** could not provide definite sterile cure (Figure 6) even upon using long-term drug administration (Figures 4 and 5).
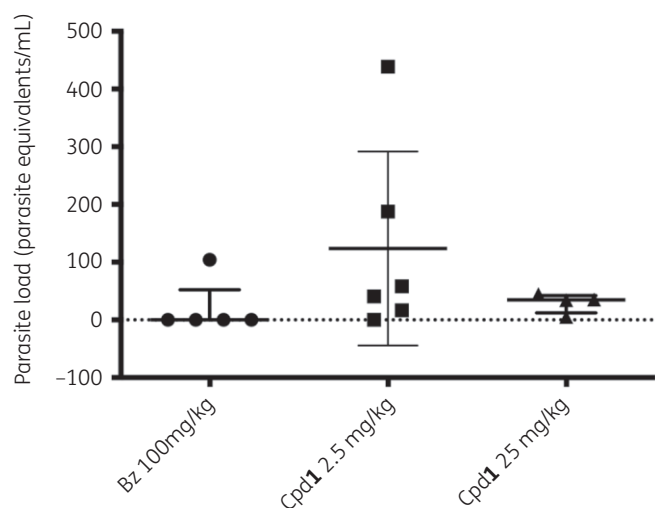
Figure 5. Blood qPCR analysis of surviving Swiss male mice infected with *T. cruzi* (Y strain) and orally treated with Cpd**1** (25 and 2.5 mg/kg, q12h) and benznidazole (Bz) (100 mg/kg, q24h). Parasite load is referred as number of parasite equivalents per mL. Each symbol on the scatter dot plots represents an individual value. Bars represent the median values and whiskers represent the IQR. Statistical analysis was performed by one-way multiple comparison ANOVA Tukey's test between the experimental groups ($P < 0.05$, 95% CI), all displaying no significant (ns) difference: $0.31 < P < 0.43$.
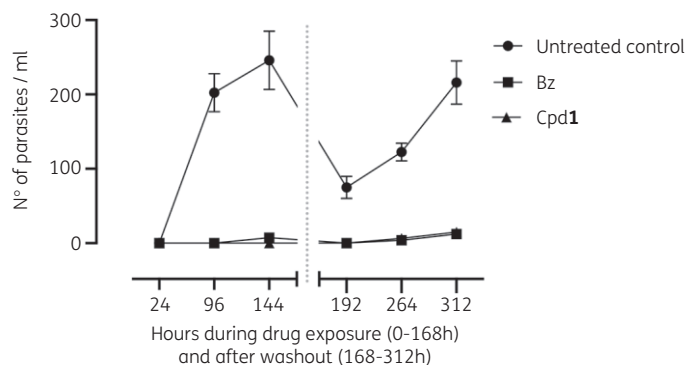
Figure 6. Washout experiments of *T. cruzi*-infected cardiac cell cultures. Number of culture-released trypomastigotes as a function of incubation time. Infected cultures (Y strain) were incubated for 168 h with Cpd**1** or benznidazole (Bz) and then another 168 h with drug-free medium before assay readout. Data represent mean $\pm$ SD of two independent experiments. The grey dotted line indicates the timepoint at which compound exposure is halted by changing to drug-free medium. Statistically significant values between control and treated groups were calculated by Welsh multiple non-parametric *t*-test ($P < 0.05$, 95% CI) and *P* values ranging $0.000009 \leq P < 0.002$.

Discussion

After more than five decades since their introduction into clinical use, the two old nitro-heterocyclic drugs benznidazole and nifurtimox still represent the only available therapeutic arsenal to treat CD patients who mostly belong to a vulnerable socioeconomic group.[34,35] This scenario has become even worse under the COVID-19 syndemic since the 30%–40% of chronic chagasic patients are more vulnerable to have a health outcome harmed or worsened by SARS-CoV-2

infection.[36] Despite these serious challenges, some recent improvement has been achieved based on the development of a paediatric formulation of benznidazole[37] and the potential adoption of a shorter benznidazole treatment regimen.[38] Fexinidazole, a 5-nitroimidazole derivative recently approved for sleeping sickness therapy[39] is under repurposing analysis in chronic chagasic patients.[40] However, no novel chemical entity has yet been discovered or implemented for this silent disease although some pre-clinical studies have reported promising drug candidates for CD, including nucleoside derivatives that act on the purine salvage pathway.[41] Our group reassessed the natural antibiotic tubercidin and a series of 7-substituted analogues aiming to identify novel hits against *Trypanosoma brucei*, the agent of human African trypanosomiasis.[42] Besides being very active against *T. brucei*, some phenyl-substituted analogues also presented high *in vitro* potency against *T. cruzi*, which motivated us to explore related 7-substituted 7-deazapurine moieties with the carbohydrate group of cordycepin (i.e. 3′-deoxy-ᴅ-ribofuranose). Some of these derivatives, such as Cpd**1,** showed an acceptable safety and efficacy profile in mouse models of *T. cruzi* infection.[23] Cpd**1** also presented desirable drug-like properties, such as good oral bioavailability and *in vitro* metabolic stability, which are essential characteristics to meet the target product profile (TPP) and allow potential clinical translation. The current criteria for 'hit' and 'lead' candidate selection for CD comprises definition of compound profile activity (preferably trypanocidal),[43] efficacy against different *T. cruzi* discrete type units (DTUs) and parasite forms relevant for human infection,[44,45] host–parasite interaction exploitation,[14] capacity to sustain sterile cure *in vitro*[43] and parasitaemia suppression and survival rates with sterile cure *in vivo*.[46] Most of these criteria were investigated for Cpd**1**, which proved highly potent against intracellular forms from different parasite strains and DTUs, displayed very high selectivity indices and was metabolically stable in the presence of mouse, human, rat and dog S9 microsomal fractions.[23] Unfortunately, no sterile cure could be achieved despite its high capacity to fully suppress parasitaemia and provide 100% survival in mouse models of *T. cruzi* infection.[23] As observed with other purine nucleoside analogues,[22,47,48] Cpd**1** was inactive against BT despite its outstanding intracellular activity in infected CC cultures ($EC_{50} = 0.029 \pm 0.006 \mu M$) and L929 cell lines ($EC_{50} = 0.25 \pm 0.17 \mu M$).[23] At that time, the lack of *in vivo* sterilization by Cpd**1** was attributed at least in part to its inefficacy towards BT and/or to the short treatment period (only 5 days) adopted in the initial *in vivo* study.

Our present study demonstrated that although Cpd**1** is not potent to induce a rapid lysis of the trypomastigotes, it induced morphological alterations (round-shape like phenotype) suggestive of impaired fitness. To check if these treated and surviving parasites were able to invade host cells and differentiate into intracellular amastigotes, a pretreatment assay on BT before infection was performed, revealing impairment of the parasite's ability to develop successfully in CC. The pre-incubation of BT with Cpd**1** followed by rinsing and quantification of the live and motile forms to normalize the parasite: host cell ratio resulted in reduced infection indices (>90%) as compared with the infected untreated parasites.

When evaluated in a mouse model of acute *T. cruzi* infection under extended drug administration (60 days), Cpd**1** could control parasitaemia but was unable to induce sterile cure as confirmed by qPCR analysis. *In vitro* washout assays corroborated these findings as Cpd**1** was unable to accomplish sterilization even though
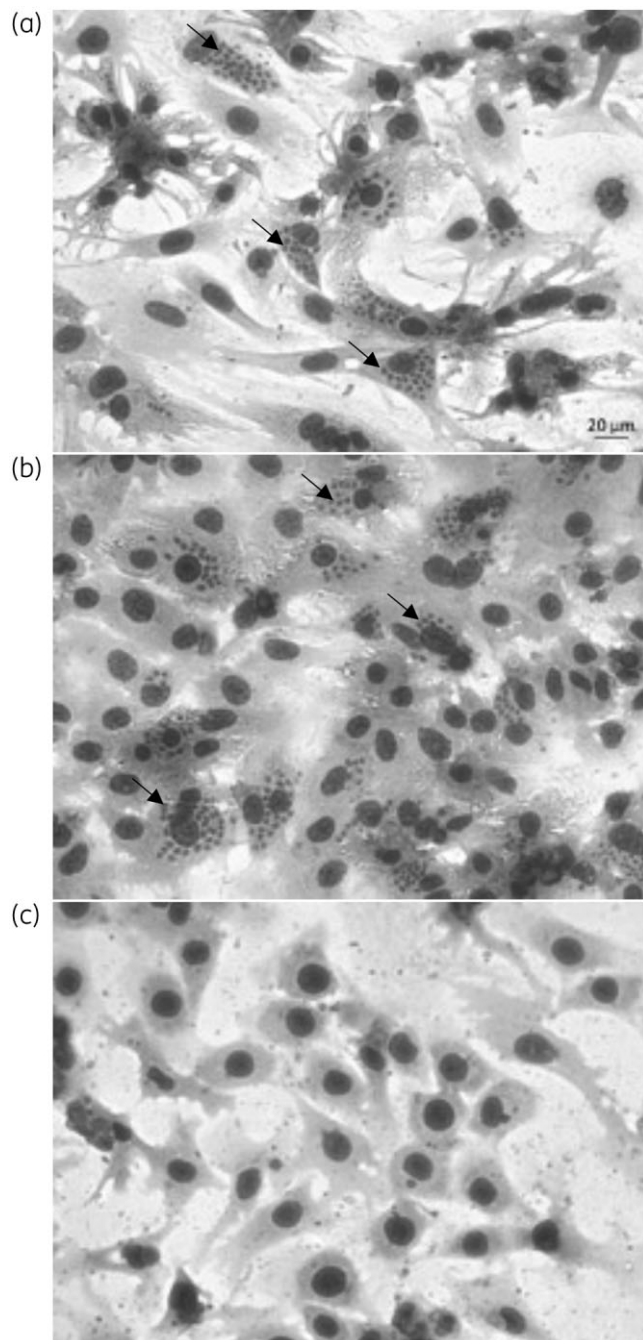
Figure 7. Light microscopy of cardiac cell cultures infected with *T. cruzi* (Y strain). (a) Untreated and treated with 1 µM (b) benznidazole (Bz) and (c) Cpd**1**. Arrows: intracellular amastigotes.

the number of released trypomastigotes was >90% lower than in untreated controls. As proposed in immunocompromised CD patients, it is probable that trypomastigotes can flourish from 'dormant forms' located in different organs, including the gastrointestinal tract.[49,50] Our present assays argue in favour of dormant forms since even though it was not able to lyse trypomastigotes, Cpd**1** impairs their ability to establish a successful *in vitro* infection. Despite outstanding efficacy against intracellular forms (Figure 7),

Cpd**1** is not able to induce *in vitro* sterilization possibly due to its inefficacy against low metabolic latent (e.g. dormant) parasites.

Drug combinations have largely been encouraged to find more efficient, shorter and safer therapies for different diseases, thereby also mitigating drug resistance.[51] For more than one decade, combinations of nifurtimox/eflornithine (NECT)[52] and sodium stibogluconate/paromomycin (SSG&PM)[53] have been successfully used for human African trypanosomiasis and cutaneous leishmaniasis, respectively, but no combination therapy is yet available for CD.[54] Our data indicated an additive interaction profile when benznidazole + Cpd**1** was used *in vitro*. *In vivo* co-administration using suboptimal doses of both agents did not improve the outcomes of each compound used separately. Conversely, an unexpected toxic profile (urination impairment) was noted that could be related to the exacerbation of renal injuries induced by *T. cruzi* infection.[55]

Our drug research complies with current criteria for drug discovery programmes of neglected tropical diseases (NTDs) caused by kinetoplastids, seeking robust proof-of-concept in preclinical trials to infer the ability of novel compounds to eliminate residual parasite nests and avoid relapses. The excellent efficacies of Cpd**1** on intracellular amastigote forms and on trypomastigote fitness were not enough to achieve *in vitro* nor *in vivo* sterilization, possibly due to the presence of dormant forms of *T. cruzi.* It remains attractive to evaluate nucleoside prodrugs to check for improved permeability and/or improved *in vivo* efficacy as novel drug candidates for NTDs like CD.

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None to declare.

Supplementary data

Figure S1 is available as Supplementary data at *JAC-AMR* Online.

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