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Successful Aspects of the Co-administration of Sterol 14 α -Demethylase Inhibitor VFV and Benznidazole in Experimental Mouse Models of Chagas Disease Caused by the Drug-Resistant Strain of *Trypanosoma cruzi*

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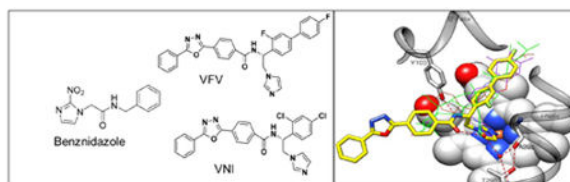
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Graphical Abstract



Keywords

Co-administration; benznidazole; VFV; VNI; Colombian strain; *Trypanosoma cruzi*; Chagas disease

Up to now, no vaccines are available for Chagas disease, and the current therapy is largely unsatisfactory. Novel imidazole-based scaffolds of protozoan sterol 14 α -demethylase (CYP51) inhibitors have demonstrated potent anti-parasitic activity with no acute toxicity. Presently our aim was to investigate the effectiveness of the experimental 14 α -demethylase inhibitor VFV in the mouse models of *Trypanosoma cruzi* infection using naturally drug-

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resistant Colombiana strain, under monotherapy and in association with the reference drug, benznidazole (Bz). The treatment with VFV resulted in complete parasitemia suppression and 100 % animal survival when administered orally (given in 10 % DMSO plus 5% Arabic gum) at 25 mg/kg (bid) for 60 days. However, as parasite relapse was found using VFV alone under this treatment scheme, the co-administration of VFV with Bz was assayed giving simultaneously (for 60 days, bid) by oral route, under two different drug vehicles (10 % DMSO plus 5 % Gum Arabic with or without 3% Tween 80). All tested mice groups resulted in >99.9 % of parasitemia decrease and 100 % animal survival. qPCR analysis performed on cyclophosphamide immunosuppressed mice revealed that, although presenting lack of cure, VFV given as monotherapy was 14-fold more active than Bz, and the co-administration of Bz plus VFV (given simultaneously, using 10 % DMSO plus 5 % Gum Arabic as vehicle) resulted in 106-fold lower blood parasitism as compared to the monotherapy of Bz. Another interesting finding was the parasitological cure in 70 % of the animals treated with Bz and VFV when the co-administration was given using the VFV suspension in 10 % DMSO + Arabic gum + Tween 80 (a formulation that we have found to provide a better pharmacokinetics), even after immunosuppression using cyclophosphamide cycles, supporting the promising aspect of the drug co-administration in improving the efficacy of therapeutic arsenal against *T. cruzi*.

Chagas disease affects more than 6 million people mostly in the poorest areas of Latin America. The available therapy is based on two nitroderivatives, nifurtimox (N) and benznidazole (Bz) that were introduced more than four decades ago and are quite unsatisfactory since both display limited activity (especially in the later chronic stage) and high toxicity. The occurrence of *Trypanosoma cruzi* strains that are naturally resistant to nitroderivatives [1] represents a special concern calling for the identification of novel trypanocidal candidates and treatment regimens. CYP51 (sterol 14 α -demethylase) is the primary target for clinical and agricultural antifungals and has been proven as a relevant target for protozoan infections [2, 3]. Previous studies revealed the high anti-parasitic efficacy of the trypanosomal CYP51 inhibitor VNI, ((*R*)-*N*-(1-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-yl)ethyl)-4-(5-phenyl-1,3,4-oxadiazol-2-yl)benzamide) against *T. cruzi* *in vitro* and *in vivo*. However, while being able to cure mice infected with the Bz-susceptible Tulahuen strain (DTU VI) [4], VNI was not able to reach high parasitological cure rates when Bz-resistant *T. cruzi* strains were employed [5], although in these experiments different drug formulations were used, which might have influenced the cure outcomes. Unfortunately, the two azoles inhibitors of fungal CYP51 (posaconazole and ruviconazole), although highly effective in pre-clinical studies, presented high levels of therapeutic failure as compared to benznidazole [6]. The lack of translation among these pre-clinical and clinical outcomes has been largely discussed, and some hypothesis raised including the need for more reproducible animal models and readouts [7, 8]. In this sense, highly sensitive *in vivo* imaging assays and *in vitro* deeper analysis of sterile cidal activity [9] claims about the limited ability of posaconazole (and other analogs) to cure *T. cruzi* experimental infections [10]. However, we can not discard the possibility that the lack of translation between clinical and pre-clinical outcomes could be due to limitations of posaconazole pharmacokinetics [11]. In fact, its maximal concentration in mice plasma does not exceed 5 μ M, and the low doses of the drug used in clinical trials for Chagas disease (because of its high cost) resulted

in 5 to 10-fold lower concentration in humans than in animal models [12, 11]. In addition to the extremely high cost of posaconazole, another important point is that fungal sterol 14 α -demethylases share less than 30 % amino acid sequence identity with the *T. cruzi* enzyme ortholog [13]. This may also explain at least in part, clinical trial failure and in turn stimulates the analysis of other inhibitors, more closely related to the protozoan enzyme, such as VNI and derivatives (5). Unlike posaconazole, this scaffold does not induce the *T. cruzi* CYP51 gene expression and does not require an increase in the dosage to sustain its anti-parasitic efficiency over time suggesting that it may have a lower propensity to induce resistance [14]. Also, due to its erratic bioavailability and unpredictable trough plasma concentration, posaconazole has been limited mainly for oropharyngeal or esophageal candidiasis and for prophylaxis in high-risk patients [15], though now, when the intravenous formulation of posaconazole has become available, its clinical use has potential to be extended [16].

Co-administration therapy has been successfully used to treat different pathologies including those triggered by parasitic infections [17, 18]. It has also been largely recommended as promising alternative therapy for CD [19] aiming to improve drug efficacy by allowing (i) to target different cellular elements and metabolic pathways, (ii) to reduce the doses and drug exposure periods thus contributing to the lowering of toxic effects, and (iii) minimizing the risk of drug resistance [20, 21]. In this regard, we investigated the anti-parasitic effect of VNI and VFV [(*R*)-*N*-(1-(3,4'-difluorobiphenyl-4-yl)-2-(1*H*-imidazol-1-yl)ethyl)-4-(5-phenyl-1,3,4oxadiazol-2-yl)benzamide] (the derivative of a broader spectrum of antiprotozoal activity [22]) in mouse models of *T. cruzi* infection caused by a highly resistant parasite strain (Colombiana) using both monotherapy and in schemes of co-administration with Bz. The *in vivo* efficacy of VFV was also investigated using different drug formulations such as DMSO with Arabic gum in the presence or not of Tween 80. Our findings demonstrate the benefits of Bz/VFV association, leading to high (>99.9 %) suppression of *in vivo* infection and reaching considerable rates of parasitological cure (70 %).

Materials and methods

Compounds

Synthesis of VNI and VFV was performed as reported [23]. In this study, the CYP51 inhibitors were diluted using (i) 10 % dimethyl sulfoxide (DMSO) plus 5 % Gum Arabic as we performed in [5] or (ii) 10 % DMSO + 5 % Arabic gum + 3 % Tween 80 – DGAT as we performed in [4, 22]. Benznidazole (Bz) was purchased from Laboratório Farmacêutico do Estado de Pernambuco, LAFEPE, Brazil and dissolved in distilled and sterile water supplemented with 3% Tween 80, which does not cause any detectable effect on mice [24]. Cyclophosphamide (Cy) (Genuxal) was purchased from Baxter Oncology (Frankfurt) and prepared in sterile distilled water [25].

Parasites

Bloodstream trypomastigote (BT) forms of Colombiana (discrete typing unit – DTU I) strain of *T. cruzi* was obtained from the blood of infected male Swiss mice at the peak of parasitemia [26]. Immediately after the purification step, the parasites were resuspended in

RPMI-1640 medium (pH 7.2–7.4) without phenol red (Gibco BRL) supplemented with 10% fetal bovine serum and 2 mM glutamine, as reported previously [27].

***In vivo* infection**

Male Swiss Webster mice were obtained from the Fundação Oswaldo Cruz (FIOCRUZ) animal facilities (ICTB /FIOCRUZ, Rio de Janeiro, Brazil). Mice were housed at a maximum of 05 per cage and kept in a standard room at 20 to 24°C under a 12 h/12 h light/dark cycle. The animals were provided with sterilized water and chow ad libitum. Mice (18 to 23 g) were infected by intraperitoneal (i.p.) route with 5×10^3 bloodstream trypomastigotes of the Colombiana strain of *T. cruzi*. Age-matched non-infected mice were maintained under identical conditions [5].

Treatment schemes

The animals were divided into the following groups (5–10 animals per group): uninfected (non-infected and non-treated); untreated (infected but treated only with vehicles); and treated (infected and treated with the compounds). The therapy was performed for 60 days starting at 10 dpi (corresponding to the parasitemia onset in this model of infection), given per oral (po) route, comparing the effectiveness of the monotherapy of each compound (100 mg/kg/day of Bz and 25 mg/kg/bid of VNI and VFV) with the simultaneous administration (60 daily doses given po, 100 mg/kg of Bz and after 15 min, 25 mg/kg of VFV, both only once a day). Only mice with positive parasitemia were used in the infected groups.

Parasitemia and mortality rates

Parasitemia was individually checked by direct microscopic counting of parasites in 5 μ L of blood, and mortality rates checked daily until 30 days post-treatment and expressed as the percentage of cumulative mortality (% CM) as described before [25].

Assessment of therapeutic failure

Mice that presented consistent negative parasitemia up to 30 days post-treatment were submitted to three cycles of cyclophosphamide exposure (50 mg/kg/day), each with four consecutive days of administration (ip) and with three days of intervals between each cycle [25]. As reported, assessment of therapeutic failure (or not) was based on the following parasitological methods: blood parasitemia reactivation observed (i) by light microscopy, and (ii) by quantitative real-time polymerase chain reaction (qPCR). Animals presenting negative results for all tests were considered “cured”. For qPCR 500 μ L blood was diluted in 1:2 volume of guanidine solution (guanidine-HCl 6M/EDTA 0.2M), and heated for 90 sec. in boiling water. Guanidine-EDTA blood samples were processed using the QIAamp DNA mini kit (QIAGEN), as described by [28]. Quantitative Real-Time PCR Multiplex assays were performed (40 cycles, threshold set at 0.01) for parasite detection targeting the *T. cruzi* satellite nuclear DNA and the internal amplification control - IAC (pZErO-2 plasmid containing an insert from the *A. thaliana* aquaporin gene), as described [29]. In uninfected samples, the result of the qPCR was NA (Non-amplified). The standard curves for absolute quantification were constructed with 1/10 serial dilutions of total DNA obtained from a

negative GEB sample spiked with 10^5 parasite equivalents per milliliter of blood (par. eq./mL).

PK studies

Pharmacokinetics and tissue distribution of VNI and VFV were studied using the previously developed protocol that is based on their oxadiazole ring-specific absorption maximum at 291 nm (ϵ_{291} 36 $\text{mM}^{-1} \text{cm}^{-1}$), which makes this inhibitory scaffold easy to monitor *in vivo*, with a detection limit of 0.1 μM (4) The compounds were given from two different formulations (3% tween 80, 10% DMSO, and 5 % Arabic gum (DGAT) or 20 % hydroxypropyl- β -cyclodextrin (HPCD)) to compare distinct administration vehicles since our previous studies revealed superior efficacy while using DGAT formulation [4,22] as compared to others vehicles [30, 5]. The compounds were given to mice by oral gavage as a single administration dose of 25 mg/kg. This dose was used in our previous studies (4,5,22,30) and was initially chosen because it is most common for testing azole drugs and drug candidates in mice. To obtain the drug plasma concentration profiles, the blood samples were collected over time (1, 2, 4, 6, 10, 16, and 24 hours after administration, using two mice per each time point), and the tissues were collected four hours after administration as we described previously [22]. For the blood analysis, 30 μl samples of plasma were diluted to 100 μl with PBS, mixed with 100 μl of acetonitrile containing 10 μM ketoconazole (used as an internal standard), the mixture was vortexed, and the drugs were extracted with 300 μl of extraction solution containing 80 % acetonitrile and 20 % water (v/v). After centrifugation at 16,000 g for 10 min the supernatant was transferred to a new tube and dried. For the analysis the samples were re-dissolved in the 500 μl of solvent composed of 50 % acetonitrile and 50 % water and analyzed using reversed-phase high-performance liquid chromatography (HPLC). The HPLC system was equipped with the dual-wavelength UV 2489 detector (Waters) set at 291 and 250 nm and a Symmetry C18 (3.5 μm) 4.6 \times 75 mm column. The mobile phase was 55 % 0.01 M ammonium acetate (pH 7.4) and 45 % acetonitrile (v/v) with an isocratic flow rate of 1.0 ml/min. For the tissue distribution analysis, prior to drug extraction approximately 100 mg of each tissue was diluted 5-fold with PBS and homogenized using an IKA Ultra Turrax T8 tissue homogenizer (The Lab World Group, USA), and 100 μl of the homogenate samples was used for the extraction and analysis as described for plasma. VFV, given from the DGAT formulation, was selected for the final set of our experiments because we have found previously that after the second administration its concentration in tissues becomes (and remains) higher than the concentration of VNI [22]. A broader spectrum of antiprotozoal activity [22,23] was another reason for selecting VFV over VNI for further co-administration studies. Finally, because VFV is detectable in plasma for more than 24 hours, it was reasonable to administer it once a day.

Ethics

The procedures dealing with infected mice and drug treatments were approved by the FIOCRUZ Committee of Ethics for the Use of Animals (CEUA LW16/14) and the procedures dealing with drug administration for the PK experiments were approved by the IACUC of Meharry Medical College.

Results

Given as monotherapy (25 mg/kg/bid, diluted in 10 % dimethyl sulfoxide (DMSO) plus 5 % Gum Arabic), VFV suppressed completely the parasitemia in infected animals, showing the effect similar to that of VNI (25 mg/kg/bid, prepared in 10 % dimethyl sulfoxide (DMSO) plus 5 % Gum Arabic) (Table 1) and Bz (100 mg/kg/day, its optimal dose) (Table 1). All treated groups reached 100 % of mice survival during the whole period of analysis (Table 1), but after the end of monotherapy, a natural parasitemia relapse was observed in all animals exposed to monotherapies of Bz and VFV but only in 3 out of five mice in the VNI group (Table 1). To confirm the therapeutic outcomes, all the animals were further immunosuppressed by 12 cycles of cyclophosphamide (Cy) and then monitored for another four weeks by counting the parasite load in the blood samples using light microscopy. We found that 20 % of the VNI group displayed a sustained negative parasitemia (which was also consistently negative according to the results of qPCR blood analysis), and VFV presented about 14-fold reduction of the blood parasite load measured by qPCR as compared to Bz (Table 2).

In the co-administration assays, the simultaneous administration of Bz and VFV (both compounds given once a day, 100 mg/kg and 25 mg/kg, respectively) revealed that besides reaching >99 % parasitemia decline at the peak and 100 % animal survival (Figure 1), two of five mice remained negative after the end of the co-administration (Table 1). After Cy administration, although none remained negative according to the light microscopy analysis, the qPCR demonstrated a substantial reduction in the blood parasite load, reaching more than 100-fold decrease in comparison with the Bz monotherapy (Table 2). In these experiments, as well as in the monotherapy experiments VFV was administered to mice using 10 % DMSO plus 5 % Gum Arabic as a vehicle, which (because of VNI and VFV hydrophobicity) was most likely not the best formulation.

Next, to compare two alternative vehicles that in our previous *in vivo* studies produced better results, we analyzed the PK of VNI and VFV given from 20% hydroxypropyl- β -cyclodextrin (HPCD) (30) or from Tween 80 (3% Tween 80, 10% DMSO, 5% Arabic gum) (DGAT) (4,22). Because the PK studies indicated a three-fold higher bioavailability when Tween 80 was added to the vehicle (Figure 2, Table 3), further studies were conducted, now using the DGAT formulation (10% DMSO + 5% Arabic gum + 3% Tween 80 (DGAT) as the vehicle. The data corroborated the high efficacy of Bz, VFV, and BZ + VFV in suppressing the parasitemia levels (> 99.8 % at the peak), leading to 100 % animal survival, while in the untreated infected group all animals died (Fig. 3, Table 4). After the drug administration, we found a natural parasitemia relapse in 6, 5 and 2 out of 10 mice for groups Bz, VFV, and the co-administration Bz plus VFV, respectively. After immunosuppression, again, we found that while Bz-treated group reached 30 % of clear blood parasite load, the co-administration resulted in 7 negative samples by qPCR (one out of 8 negative samples was lost during the qPCR procedure), thus reaching 70 % of cure when Bz + VFV were given using DGAT vehicle (Table 4).

Discussion

There is an urgent need for safer and more efficacious drugs for Chagas disease, a neglected silent life-threatening illness that causes more than 7,000 annual deaths, with about 25 million people at risk of infection in endemic areas of Latin America, affecting mainly rural populations in areas of low resources, where human contact with the vectors is frequent [31]. Despite the fact that vector control and blood transmission regional initiatives have successfully achieved substantial reduction in the number of new acute cases [32], other concerns still remain as challenges, including the disease globalization, the existence of alternative routes of transmission, such as mother-to-child or oral transmission through contaminated food [33, 34], and particularly the high variability in the drug sensitivity across multiple strains that comprise the genetically diverse population of *T. cruzi*. Therapeutic failures are well documented in Chagas disease under the use of Bz and nifurtimox, which seems to depend on the interplay of the genetic background of the *T. cruzi* strains and their mammalian hosts, the drug access and accumulation in different environments, and the host immune response [35, 36].

Thus, as the available chemotherapy for CD has substantial limitations, diverse pre-clinical approaches have been used in an attempt to identify new therapeutic alternatives, including the screening of natural and chemical libraries [37], the repositioning of drugs already licensed to other diseases [38], the synthesis and validation of specific inhibitors targeting parasite molecules [39], as well as the use of co-administration of licensed drugs with promising novel candidates [19, 21]. Among the target-directed anti-parasitic approaches, inhibitors of the sterol biosynthesis pathway, in particular, those related to the CYP51 enzyme, have been widely studied on experimental infection with *T. cruzi* [21, 40] and two of them, the antifungal drug posaconazole and the prodrug of another antifungal drug candidate ravuconazole (E1224) have been recently moved to clinical trials but unfortunately displayed limited treatment success in chronic chagasic patients [41].

Because we found previously that VNI, our experimental inhibitor of Tulahuen *T. cruzi* CYP51, though suppressing the parasitemia and preventing mortality, was unable to achieve the complete parasitological cure in the mouse models of Colombian *T. cruzi* infection (20 day treatment, 25 mg/kg/bid, administered using 10 % DMSO plus 5 % Arabic gum) [5], in this study we mostly concentrated on the CYP51 structure-based VNI derivative VFV, since VFV has a broader spectrum of activity as the protozoan CYP51 inhibitor and was shown to be more potent than VNI against the Y strain *T. cruzi* [42] and Leishmania [22].

The present studies were conducted employing a longer period of treatment (60 days) using Bz (as reference drug at its optimal dose (100 mg/kg/day) for mouse *T. cruzi* infection [5, 25]) and the imidazole molecules (VNI and VFV) under monotherapy regimen and also addressing the scheme of drug co-administration (using two different vehicles, 10 % dimethyl sulfoxide (DMSO) plus 5 % Arabic gum [5] and 10% DMSO + 5% Arabic gum + 3% Tween 80 – DGAT. The later drug – formulation was chosen to improve pharmacological status as our PK studies demonstrated higher bioavailability and tissue distribution when Tween was added to the vehicle solution. plus 5 % Arabic gum) was more effective than Bz in reducing the blood parasitism measured by qPCR analysis of blood

samples from treated mice submitted to Cy injection. VFV resulted in more than 14-fold reduction in blood parasite DNA that corresponds to lower and subpatent blood parasitism. Another significant finding was the improved efficacy of VFV when administrated in co-administration with Bz resulting in >100-fold less blood parasitism measured by qPCR than Bz alone. Interestingly, exposure to Bz plus VFV induced 70 % blood parasite clearance when the second vehicle was used (10% DMSO + 5% Arabic gum + 3% Tween 80 – DGAT), supporting the PK studies that demonstrated higher plasma levels and better tissue distribution of VFV when Tween 80 is added to the vehicle solution and corroborating the previous findings using acute and chronic *T. cruzi* mouse infection (Tulahuen strain) that demonstrated high rates of cure when these CYP51 inhibitors were given using DGAT solution [4]. Also, the promising aspect of this co-administration drug scheme reinforces the potential use of CYP51 inhibitors along with Bz as therapeutic arsenal against *T. cruzi*. Other studies also revealed the successful use of drug co-administration in experimental animal models of *T. cruzi* infection, e.g., amidine compounds and Bz [25] and also other compound classes including posaconazole and Bz (24,40,41,42). The advantages of VFV include its low cellular ($EC_{25} > 50 \mu\text{M}$, NIH/3T3 [23]) and *in vivo* toxicity (NOAEL > 200 mg/kg [30]), weak influence on the major human drug-metabolizing cytochromes P450 (e.g., CYP3A4 $IC_{50} = 3.6 \mu\text{M}$ vs. $IC_{50} = 0.14 \mu\text{M}$ for posaconazole), moderate hepatic clearance, and the ability to penetrate different tissues and organs [23].

To summarize, this study supports the use of co-administration schemes of anti-parasitic drugs as future potential clinical evaluation of Chagas disease. The curative action of Bz/VFV co-administrations was achieved in a well-established acute infection model with the Colombiana strain using optimal doses of compounds that did not exert any signs of animal toxicity until the endpoint (>120 days of following up). These findings accumulate evidence that it is possible to achieve a better therapeutic outcome using Bz in co-administration with other drug candidates that target other metabolic pathways of the parasite. Several other pathogen-induced diseases such as tuberculosis, leprosy, HAT and HIV infection reached better efficacy with co-administration of drugs with different mechanisms of action. The co-administration drug treatment not only can boost the effect of the different therapeutic compounds but may also aid in avoiding the development of parasite chemotherapeutic resistance [19]. Indeed, more than one new drug is needed for each so that co-administration can be employed to avoid drug resistance and to provide back-up drugs when resistance emerges [34]. The presented results encourage further investigations aiming to achieve therapeutic and affordable orally administrated drugs, as alternatives for those millions of chagasic patients.

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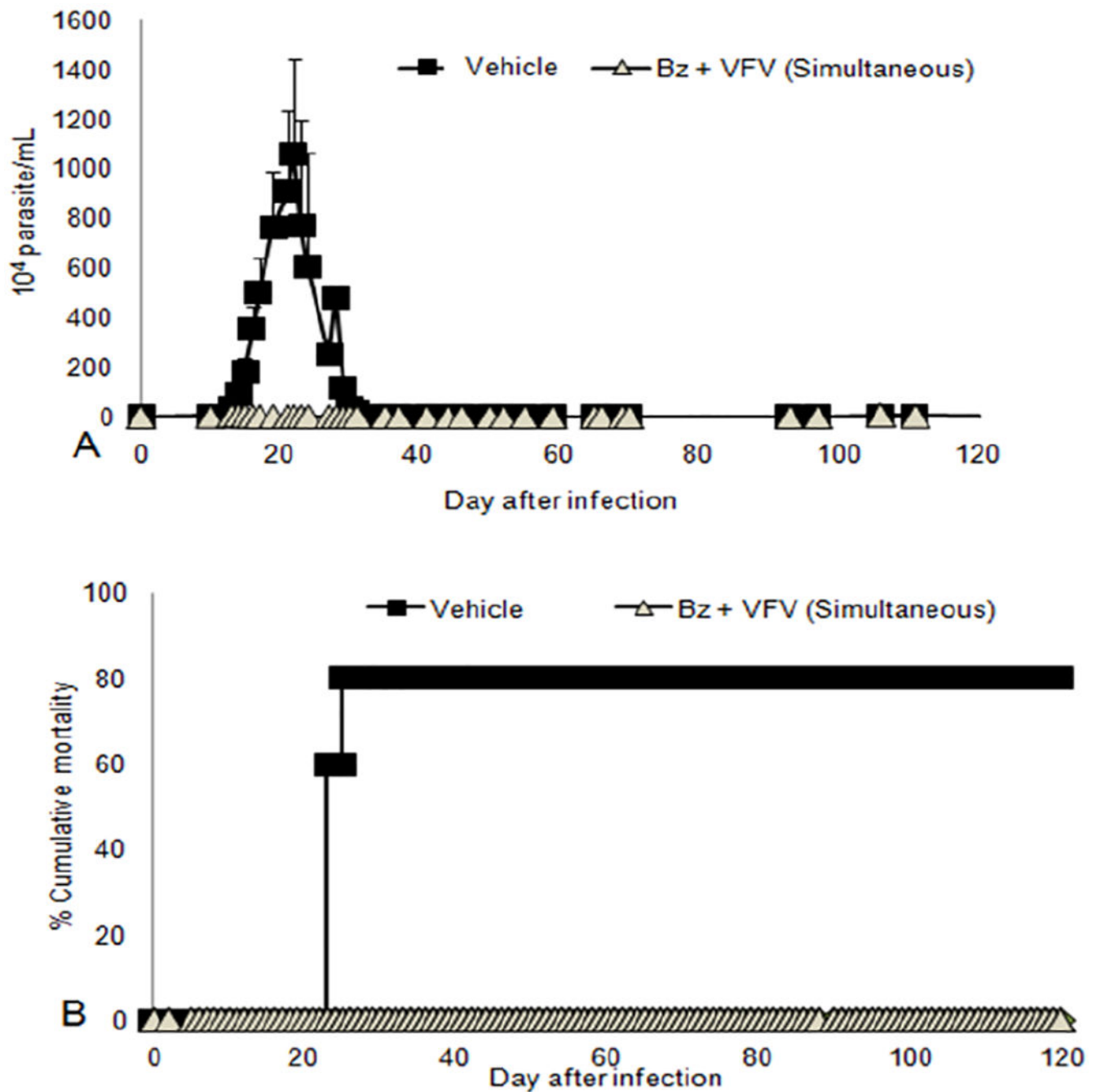


Figure 1: Effect of VFV (25 mg/kg/day, in 10 % dimethyl sulfoxide (DMSO) plus 5 % Gum Arabic administered simultaneously with Benznidazole (Bz) – 100 mg/kg/day) for 60 days in the experimental mouse model of Chagas disease caused by the Colombian strain of *Trypanosoma cruzi* (n=5). (A) Parasitemia levels and (B) Percentage of cumulative mortality.

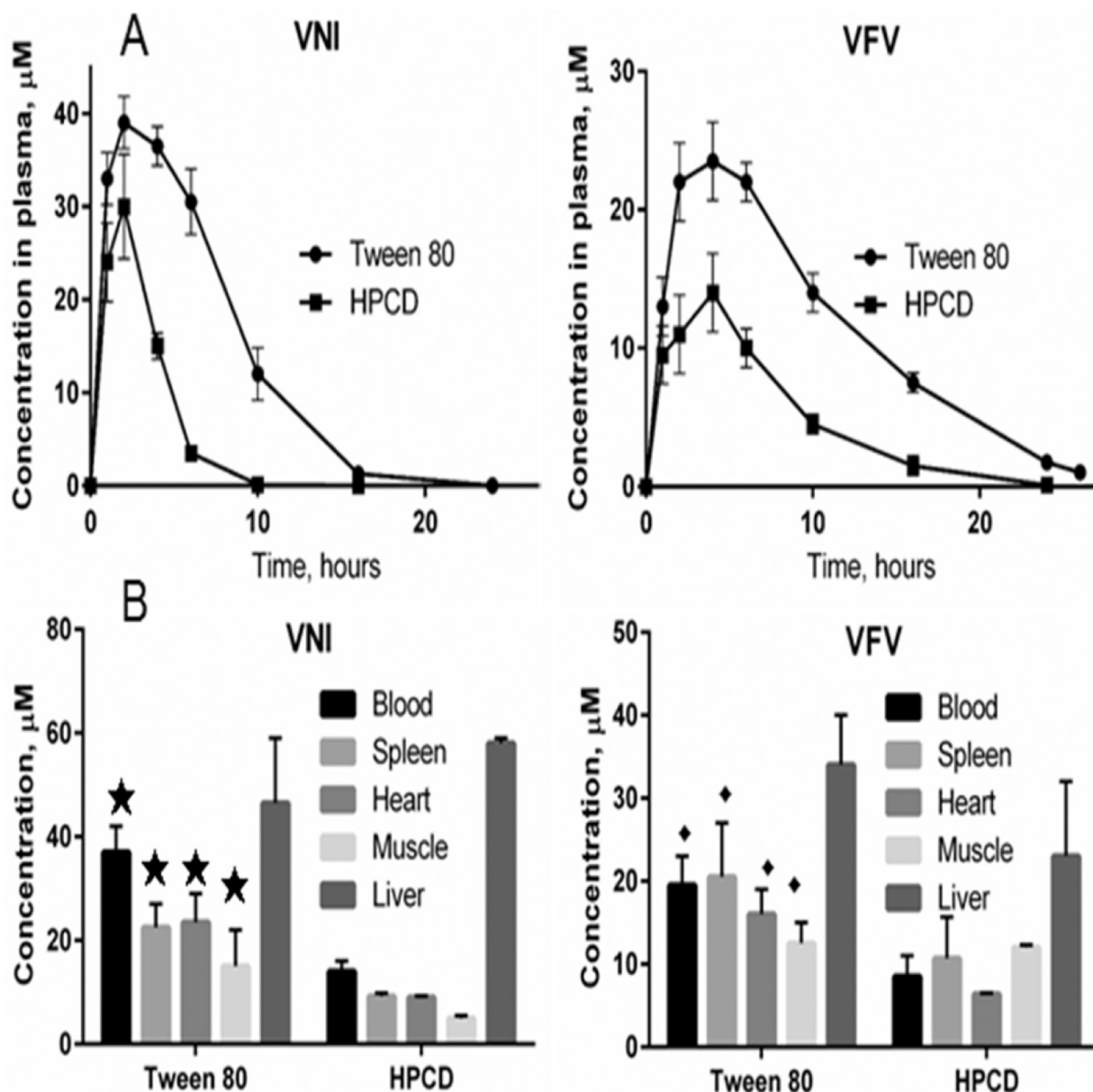


Figure 2: Pharmacokinetics of VNI and VFV in mice after a single oral administration dose of 25 mg/kg depending on formulation: DGAT (3% Tween 80, 10% DMSO, 5% Arabic gum) or HPCD (20% hydroxypropyl- β -cyclodextrin). 16 mice were used for testing of each drug and two mice were used for each of the 8 independent experimental points. **A.** Plasma concentration profiles. **B.** Tissue distribution 4 hours after administration. Panel A, $p < 0.001$ between VNI/DGAT and VNI/HPCD; and $p < 0.0015$ between VFV/DGAT and VFV/HPCD. Panel B, $\star p < 0.015$; $\blacklozenge p < 0.02$ (Two-Way-Anova).

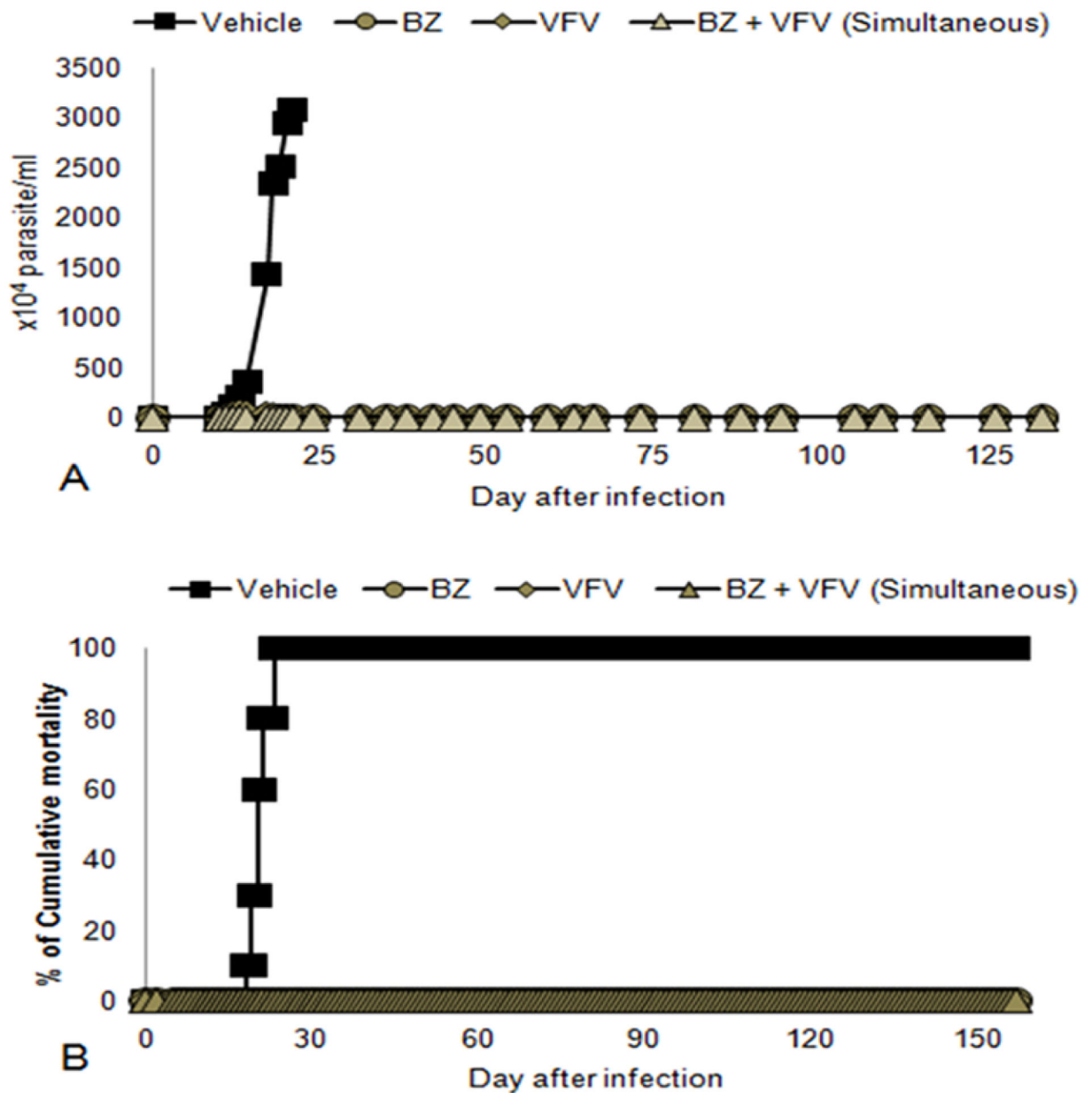


Figure 3: Effect of VFV (25 mg/kg/day) administered simultaneously with Benznidazole (Bz) - 100mg/kg/day for 60 days in the experimental mouse model of Chagas disease caused by the Colombian strain of *Trypanosoma cruzi* (n=10). (A) Parasitemia levels and (B) Percentage of cumulative mortality. VFV was diluted in 10 % DMSO + 5% Arabic gum + 3 % Tween 80 – DGAT.

Swiss male mice infected with bloodstream trypomastigotes of *Colombiana* strain (n=5) and submitted to mono (VFV, VNI and Bz) and co-administration therapy (Bz and VFV). VNI and VFV were diluted in 10 % DMSO plus 5 % Gum Arabic.

Table 1 -

| | Parasitemia peak (mean \pm SD number parx10 ⁴ /mL) | % Parasitemia suppression | % Animal survival | Natural relapse after the therapy |
|-------------------------|---|---------------------------|-------------------|-----------------------------------|
| Bz 60 days | 0 \pm 0 | 100 | 100 | 5 out of 5 mice |
| VNI 60 days | 0.2 \pm 0.4 | 99.9 | 100 | 3 out of 5 mice |
| VFV 60 days | 0.2 \pm 0.4 | 99.9 | 100 | 5 out of 5 mice |
| Bz+VFV (simultaneous)** | 1.4 \pm 3.1 | 99.9 | 100 | 3 out of 5 mice |

** Simultaneous therapy: 60 days of Bz + VFV (once a day)

qPCR analysis of Swiss male mice infected with bloodstream trypomastigotes of Colombian strain (n=5) and submitted to co-administration therapy (Bz and VFV). VFV was diluted in 10 % DMSO plus 5 % Gum Arabic.

Table 2 -

| | % Negative blood qPCR* | Blood parasite load determined by qPCR |
|-------------------------|------------------------|--|
| Bz+VFV (simultaneous)** | 0 | 123 ± 53 |
| Bz | 0 | 13038 ± 13612 |
| VNI | 20 | 372 ± 528 |
| VFV | 0 | 905 ± 854 |

* Surviving mice

** Simultaneous therapy: 60 days of Bz + VFV (once a day)

Table 3 -

PK profile data for VNI and VFV given from HPCD and DGAT formulations.

| Compound | Formulation | T _{max} , hours | C _{max} , μM [μg/mL] | AUC, μg × h/L | Life time, hours ^a |
|----------|-------------|--------------------------|-------------------------------|---------------|-------------------------------|
| VNI | DGAT | 2 | 39±2 [20] | 163 | 16 |
| | HPCD | 2 | 30±4 [15] | 55 | 10 |
| VFV | DGAT | 4 | 24±2 [11] | 159 | >26 |
| | HPCD | 4 | 14±2 [8] | 64 | <24 |

^aLife time, hours: The time until compound becomes undetectable given by per oral route (below 0.1 μM, which is the detection limit). References: 4 and 22.

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Table 4 -

Swiss male mice infected with bloodstream trypomastigotes of *Colombiana* strain (n=10) and submitted to mono (VFV and Bz) and co-administration therapy (Bz and VFV). VFV was diluted using 10 % DMSO + 5 % arabic gum + 3 % Tween 80 - (DGAT) and Bz dissolved in water + 3 % Tween 80.

| | Parasitemia peak (mean \pm SD number parx10 ⁴ /mL) | % Parasitemia suppression | % Animal survival | Natural relapse after the therapy | % Blood parasite clearance (negative qPCR) |
|-------------|---|---------------------------|-------------------|-----------------------------------|--|
| Bz 60 days | 0 \pm 0 | 100 | 100 | 6 out of 10 mice | 30 |
| VFV 60 days | 5.7 \pm 5.1 | 99.8 | 100 | 5 out of 10 mice | 0 |
| Bz+VFV | 0 \pm 0 | 100 | 100 | 2 out of 10 mice | 70* |

* One out of the 8 negative samples was lost during the qPCR procedure