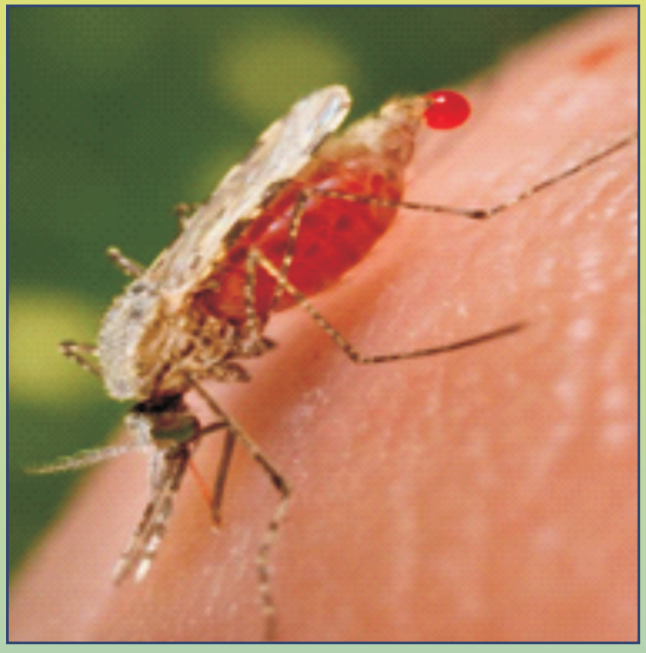
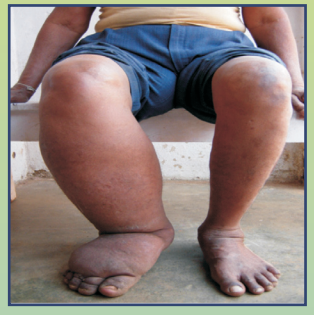
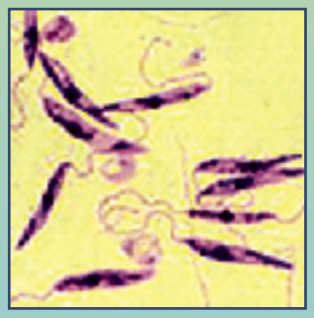
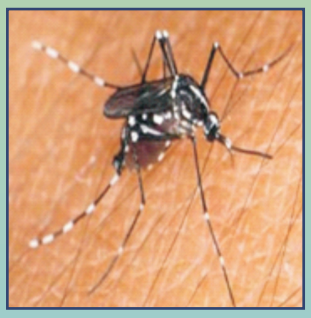
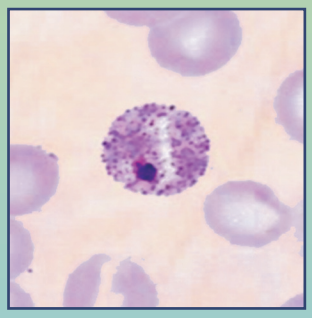
Volume 56 Number 3
September 2019

ISSN 0972-9062
Coden JVBDAO

Journal of Vector Borne Diseases

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J Vector Borne Dis 56, September 2019, pp. 237–243

Nifurtimox response of *Trypanosoma cruzi* isolates from an outbreak of Chagas disease in Caracas, Venezuela

Arturo Muñoz-Calderón[1], Zoraida Díaz-Bello[1], José Luis Ramírez[2], Oscar Noya[3–4] & Belkisyolé Alarcón de Noya[1]

[1]*Sección de Inmunología, Instituto de Medicina Tropical, Facultad de Medicina, Universidad Central de Venezuela (IMT-FM-UCV), Caracas;* [2]*Centro de Biotecnología, Fundación Instituto de Estudios Avanzados, Caracas;* [3]*Sección de Biohelmintiasis, IMT-FM-UCV, Caracas;* [4]*Centro para Estudios sobre Malaria, Instituto de Altos Estudios "Dr. Arnoldo Gabaldón", Instituto Nacional de Higiene 'Rafael Rangel, Ministerio del Poder Popular para la Salud, Caracas, Venezuela*

ABSTRACT

Background & objectives: In Venezuela, Chagas disease (ChD) is considered a serious health problem, with about 6 million people at risk; and acute outbreaks due to oral transmission of Chagas Disease (OChD) are becoming increasingly important. In 2007 there was a major outbreak of OChD and although patients from this episode were treated with nifurtimox (Lampit®—Bayer), about 70% therapeutic failure was registered. These results led us to examine whether parasite's drug susceptibility was related to this therapeutic failure.

Methods: The *Trypanosoma cruzi* parasites were isolated by haemoculture of the peripheral blood drawn from the pre- and post-nifurtimox treated patients infected in the 2007 OChD outbreak at Caracas, Venezuela. The *in vitro* assays for drug testing were performed by the MTT methodology followed by calculation of inhibitory concentration-50 (IC_{50}) values.

Results: Parasite isolates obtained from the infected patients prior and after nifurtimox treatment when subjected to variable concentrations of the drug showed great heterogeneity in susceptibility with IC_{50} values ranging from 4.07 ± 1.82 to 94.92 ± 7.24 μM.

Interpretation & conclusion: The high heterogeneity in nifurtimox IC_{50} values in the isolates and clones from the OChD patients, suggests that the therapeutic failure to nifurtimox could be due in part to a phenotypic variability that existed in the wild parasite population at the original source of contamination. Though, further pharmacological studies are needed to confirm the existence of natural nifurtimox resistance in the parasite.

Key words Caracas; Chagas disease; nifurtimox; oral transmission; resistance; susceptibility assay; *Trypanosoma cruzi*

INTRODUCTION

The protozoan *Trypanosoma cruzi* is the etiological agent of Chagas disease (ChD), which ranks among the world's most neglected diseases. According to the estimates of the Pan American Health Organization and the World Health Organization (WHO) there are 6 to 7 million people infected with *T. cruzi* worldwide with the great majority being in Latin America[1–2].

In Venezuela, nearly 6 million people living in 198 municipalities and 14 federal entities are considered to be at risk[3]. In humans, although the main route of *T. cruzi* infection is through the contamination of skin wounds with triatomines faeces containing metacyclic trypomastigotes; other modes of transmission, such as blood transfusion, organ transplantation, congenital, laboratory accidents and oral transmission have also been documented[4–5]. Given a large number of reports of acute outbreaks of oral Chagas disease (OChD) in the last decade, the importance of the type of transmission is increasing[6–9].

Since 2007 when we reported the world's first largest oral transmission, 14 outbreaks of OChD have been documented in Venezuela[8,10–11]. In the 2007 outbreak, medical and parasitological monitoring of nifurtimox treated-patients was not entirely satisfactory, and after eight years of treatment, about 70% of the patients showed persistent IgG, lytic antibodies, positives PCRs for kinetoplast DNA markers, and in some cases circulating parasites in the peripheral blood[11]. The reasons for treatment failures are unknown, but among the potential causes are variable drug susceptibility in *T. cruzi* populations, characteristics of the host's immune system and/or unfavourable drug pharmacokinetics properties[12]. Since differences in drug susceptibility have been experimentally determined in *T. cruzi* isolates from different geographic areas[13–20], we believe that *T. cruzi* populations naturally resistant to benznidazole and nifurtimox can be considered as an important factor behind the low cure rates observed in that outbreak[15–17].

This study for the first time describes the experimental susceptibility/resistance of *T. cruzi* parasites to nifurtimox in pre- and post-treated patients infected with OChD during the 2007 outbreak using trypomastigotes–the flagelated stage of trypanosomes found in peripheral blood.

MATERIAL & METHODS

Patient and parasite isolates

This study was conducted in 2018 (January to November) involving OChD patients of the 2007 Chacao (city in Caracus) outbreak, diagnosed and treated at the Section of Immunology, Institute of Tropical Medicine in Caracas, Venezuela. Patients were administered nifurtimox orally at doses of 8 mg/kg/day, divided into two doses for 90 continuous days. The clinical follow-up ensured the compliance of the treatment in all the patients.

Seven pre-treatment (Pre-Tt) and four post-nifurtimox treatment samples isolated from peripheral blood from different patients were used. The isolates of *T. cruzi* were obtained by haemoculture (Hm) following the method proposed by Filardi and Brener[16] and preserved in liver infusion-tryptose (LIT) medium supplemented with 10% fetal bovine serum (FBS) and 10% dimethyl sulfoxide (DMSO) at –194 °C. Two international reference isolates were used as controls: Dm28c, naturally resistant to nifurtimox[21] and Cl-Brener, naturally susceptible to nifurtimox[22]. Their characteristics are shown in Table 1.

In vitro host cell line

For *in vitro* cell infections we used African green monkey kidney epithelial cells (Vero cell-ATCC), kindly donated by the Instituto Nacional de Higiene "Rafael Rangel" (INHRR, Caracas-Venezuela). Cells were cultured in minimal essential medium (MEM) supplemented with 5% FBS and incubated at 37 °C in an atmosphere of 5% CO_2.

Table 1. General characteristics of *Trypanosoma cruzi* isolates from oral Chagas disease patients

Isolate code	Biological origin	Status	DTU	Date of isolation
1593*	Human	Pre-treatment	I	December 2007
1595**	Human	Pre-treatment	I	December 2007
1601**	Human	Pre-treatment	I	December 2007
843.1***	Human	Post-treatment	I	April 2008
843.2***	Human	Post-treatment	I	May 2008
909***	Human	Post-treatment	I	June 2008
875***	Human	Post-treatment	I	July 2008
Dm28c	*Didelphis marsupialis*	International reference strain	I	–
Cl-Brener	*Triatoma infestans*	International reference strain	VI	–

DTU– Discrete typing units; (*) – Reference no. 23; (**) – Reference no. 24; and (***) – Reference no. 24.

Cellular infection

Trypomastigote stages derived from each isolate were differentiated from aged epimastigotes culture in a 10:1 ratio (aged epimastigote: Vero cells). When trypomastigotes started to emerge from Vero cells, the concentration of living parasites was determined using a Neubauer chamber.

Parasite cloning

Clones from the isolates listed in Table 1 were obtained from the trypomastigotes released from Vero cells using the methodology described by Goldberg and Chiari[26] with some modifications. A single trypomastigote, isolated by serial dilution, and assessed by light microscopy, was placed in each of the 96-well plates containing approximately 500 Vero-ATCC cells per well. The plate was incubated at 37 °C in a 5% CO_2 atmosphere and followed up for 15–20 days until the parasite´s growth was observed. Free trypomastigotes were then transferred to cell culture flasks with 70% confluent Vero-ATCC cells, and further cultured in blood agar + LIT medium supplemented with 10% inactivated FBS and 1% of the penicillin-streptomycin solution. Two clones were generated for each parasite isolate.

Reagents

Nifurtimox is a commercial drug from Bayer Laboratories (Germany). The 100 mg of this drug was dissolved in DMSO, keeping DMSO final concentration not exceeding 1% v/v. Before each test, drug dilutions were prepared in MEM.

In vitro assays for drug testing

About 100 µl of trypomastigotes were added at a concentration of 1×10^8 parasites/ml to each of the 96-wells of a flat bottom plate. Immediately 50 µl of nifurtimox was added at various concentrations (0.0348, 0.348, 3.480, 34.80, 348 µM). Plates were incubated at 37 °C for 24 h. After the incubation, 20 µl of tetrazolium salt (MTT) was added to each well. Plates were incubated for 3 h at 37 °C, and the formazan crystals were dissolved with a solution of 0.01% SDS in 0.1N HCl. The formazan crystals were quantified with a TECAN-Sunrise microplate reader at 570 nm. Each assay was done in triplicate with their respective controls without the drug[24].

Statistical analysis

Drug activity was expressed as the concentration necessary to inhibit parasitic growth by 50% (IC_{50})[24–27]. A 95% confidence interval was calculated using nonlinear regression with GraphPad Prism (Intuitive Software for Science, San Diego, CA, USA) software. The results are shown as the mean ± standard deviation (SD) of three independent experiments.

Ethical statement

In order to obtain isolates of *T. cruzi* from peripheral blood of adults and children, patients signed an informed consent form, approved by the Ethics Committee of the Institute of Tropical Medicine, Faculty of Medicine, Universidad Central de Venezuela (CEC-IMT 019/2010; December 10, 2010) .

RESULTS

In vitro nifurtimox cytotoxicity on parental populations of Trypanosoma cruzi: Pre- and post-treatment

As shown in Table 2, nifurtimox susceptibility of OChD *T. cruzi* isolates from the 2007 Chacao outbreak was very heterogeneous with IC_{50} values ranging from 4.07 ± 1.82 µM and 94.92 ± 7.24 µM. Figure 1 shows the survival curves of the isolates listed in Table 1 after 24 h exposure to different concentrations of nifurtimox.

Table 2. Nifurtimox inhibitory concentration$_{50}$ (IC_{50}) values obtained from *Trypanosoma cruzi* from patients with oral Chagas disease of the Chacao Municipality outbreak in 2007

Isolates/ strain	Isolate code	IC_{50} (µM) of isolated parental	Clone	IC_{50} (µM) of clones	Biological stage	IC_{50} (µM) of Ref. strain
Pre-treatment	1593	23.31 ± 3.24	1593-C1	99.12 ± 0.82	Trypomastigote	NA
			1593-C2	0.03 ± 0.009		
	1595	64.40 ± 8.42	1595-C1	ND		NA
			1595-C2	ND		
	1601	22.35 ± 2.87	1601-C1	98.31 ± 4.57		NA
			1601-C2	ND		
Post-treatment	843.1	4.07 ± 1.82	843.1-C1	210.76 ± 10.26		NA
			843.1-C2	2.92 ± 1.28		
	843.2	43.31 ± 4.14	843.2-C1	43.29 ± 5.94		NA
			843.2-C2	284.32 ± 6.83		
	909	30.39 ± 3.31	909-C1	736.59 ± 15.04		NA
			909-C2	721.19 ± 8.79		
	875	94.92 ± 7.24	875-C1	ND		NA
			875-C2	ND		
Ref.	CL-Brener	4.51 ± 0.34	NA	NA		#
	Dm28c	19.19 ± 1.54	NA	NA		10.0 ± 0.4

Ref.: International reference strain[28]; ND: Not determined; NA: Not applicable; #No reference values found.

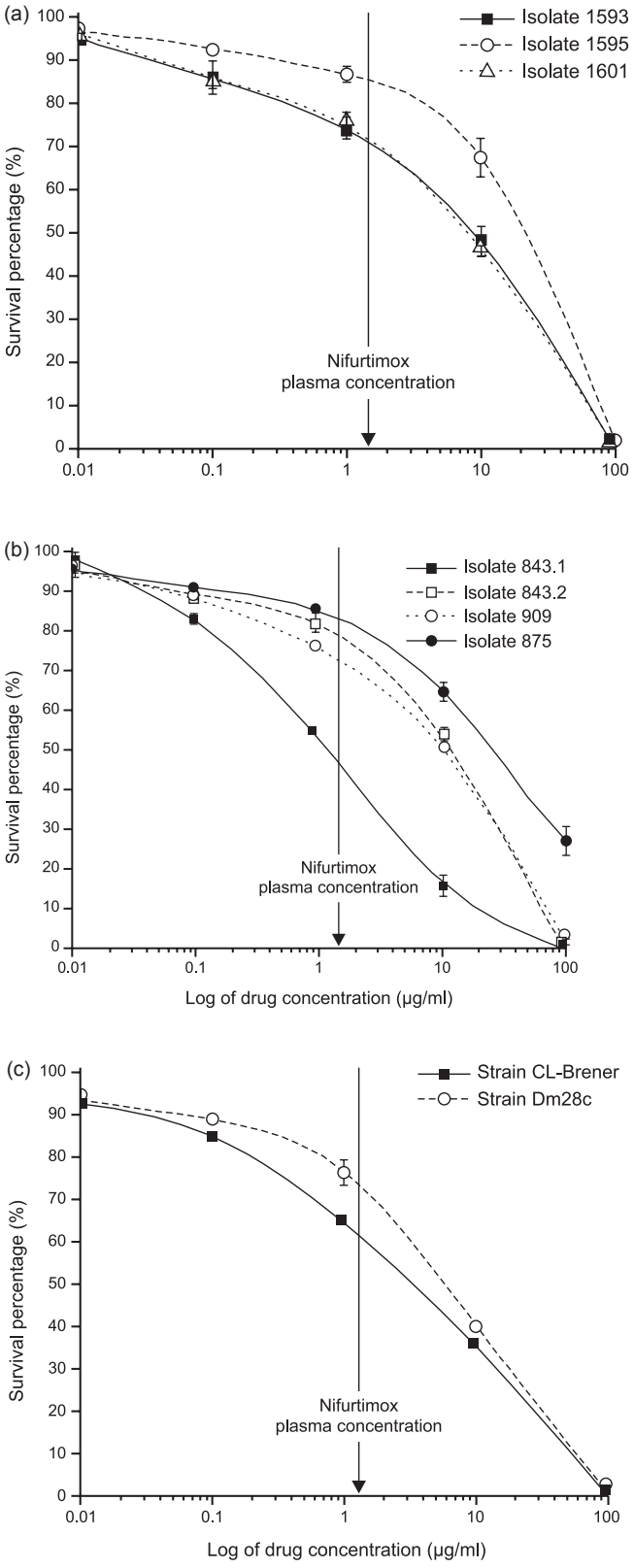

Fig. 1: Survival curves for *Trypanosoma cruzi* trypomastigotes treated with nifurtimox after 24 h incubation: (a) Pre-treatment isolates; (b) Post-treatment isolates; and (c) Strains of international reference Dm28c (naturally resistant to nifurtimox) and Cl-Brener (naturally susceptible to nifurtimox).

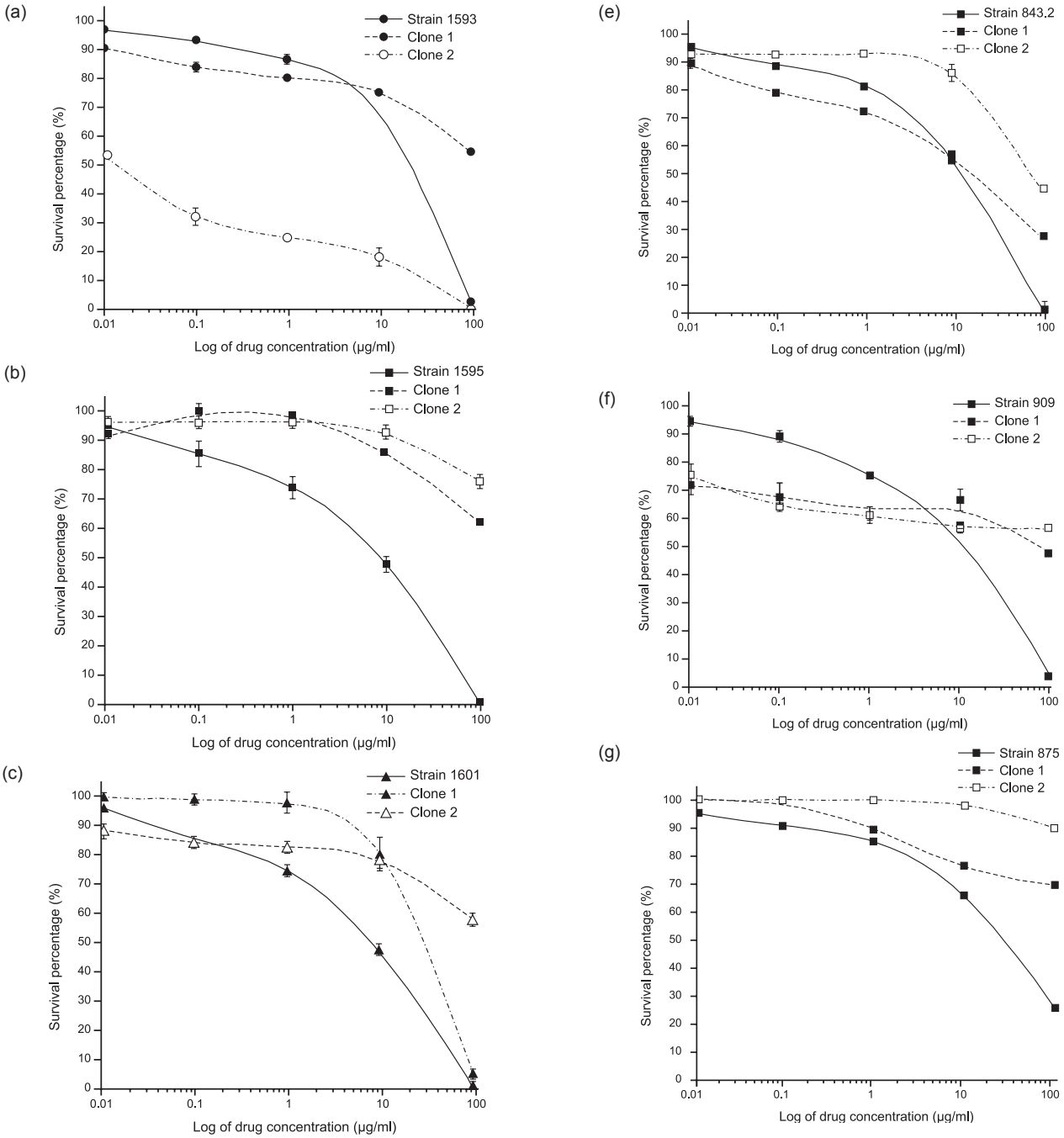

Fig. 2: Dose-response of clones derived from the original parental isolates. Pre-treatment: (a) 1593; (b) 1595; (c) 1601; Post-treatment: (d) 843.1, (e) 843.2, (f) 909, (g) 875.

In the case of the Pre-Tt isolates (Fig. 1a) at 100 µg/ml (348 µM), a 100% cytotoxic effect was achieved in all isolates. However, IC_{50} values revealed heterogeneity in the results, showing up to three times more susceptibility in isolates 1601 and 1593 (22.35 and 23.31 µM, respectively) than isolate 1595 (64.40 µM).

The estimated average survival for the Pre-Tt isolates at a nifurtimox concentration of 4.5 µM, which is the plas-

ma concentration after an extravascular administration of the drug, was approximately 85%, *i.e.* a maximum efficiency close to 15%.

Regarding nifurtimox cytotoxicity in Post-Tt isolates (Fig. 1b), sample 875 did not reach a 100% cytotoxic effect at the largest nifurtimox dose (348 μM). Pre-Tt samples did not show a cytotoxic effect greater than 50% at the plasma concentrations of nifurtimox (4.5 μM). These corroborate the presumed resistant phenotype observed in the pre-Tt populations.

Isolate 843.1, was the only one not following this trend, showing survival of around 50% with an IC_{50} of 4.07 ± 1.82 μM. These values can be considered as nifurtimox susceptible when compared with the resistant reference strain Dm28c (Fig. 1c).

In order to check isolates phenotype stability, the nifurtimox assays were done a week apart in triplicate. Additionally, nifurtimox sub-cultures susceptibility after a follow up of eight months showed no significant variation (results not shown).

An interesting finding emerged when *in vitro* assays were performed to evaluate the nifurtimox resistance in two independent samples isolated from the same patient a month apart after receiving nifurtimox treatment at 8 mg/kg for 90 days. These two Post-Tt isolates designated 843.1 and 843.2 showed different susceptibility to the nifurtimox (Fig. 1b) with IC_{50} values of 4.07 ± 1.82 μM and 43.31 ± 4.14 μM, respectively*, i.e*. the 843.1 isolate presented a 10-fold reduction in nifurtimox susceptibility. Figure 1b also reveals that at the plasma concentration of nifurtimox (4.5 μM), 843.2 isolate showed approximately a 2-fold higher survival than isolate 843.1.

In vitro nifurtimox cytotoxic effect in cell clones derived from parent T. cruzi isolates from the pre and post-treatment patients with oral Chagas disease

Figure 2 showing the survival curves for different clones from the same parental stock display high heterogeneity in IC_{50} values. The IC_{50} overall variation among clones ranged from 0.03 ± 0.009 μM (clone 2 from isolate 1593) to 736.59 ± 15.04 μM (clone 1 from isolate 909). The greatest variability to nifurtimox was observed in clones from isolates 1593 (Fig. 2a), 909 (Fig. 2f) and 875 (Fig. 2g), where IC_{50} differences among clones from the same isolate varied up to 80 fold in nifurtimox susceptibility. In the case of clone 2 from isolate 875, a 100% decrease in sensitivity was observed at the plasma concentration of the drug, whereas in clone number 2 from isolates 1593 (Fig. 2b) and 843.1 (Fig. 2d) the sensitivities to the drug were higher with IC_{50} values of 0.03 and 2.92 μM, respectively. These IC_{50} values are up to 150 times smaller than the values obtained for the CL-Brener strain (control strain for susceptibility).

DISCUSSION

The natural resistance of *T. cruzi* populations to nitroderivatives is described as an important factor that could explain therapeutic failure in humans and experimental models. Although these compounds have been used for decades in the treatment of Chagas disease, the biological behavior of their components on the parasitic stages of *T. cruzi* remains poorly understood. Here we present one of our few works in which *T. cruzi* trypomastigotes forms have been used as experimental models. These trypomastigotes were obtained from patients affected by OChD pre- or post-treatment with nifurtimox. In general, the parasite populations isolated from these patients showed a natural resistance to nifurtimox. The IC_{50} values for some isolates were similar or larger than the resistant strain Dm28c. This result is similar to the IC_{50} values of 10.00 ± 0.4 μM and 17.4 ± 5.1 μM reported by Vázquez-Rodríguez *et al*[28] for trypomastigotes and epimastigote forms, respectively.

In the case of Pre-Tt isolates 1601 and 1595, we found a 5- to 14.3-fold increase in the IC_{50} values when compared with the reference strain Dm28c, thus revealing that the parental nifurtimox resistant phenotype was present in the original inoculum. Some authors have reported that within *T. cruzi* DTUI isolates from the wildlife cycle have a natural resistance to nifurtimox[29].

Comparing the Pre-Tt isolates with the Post-Tt clones we registered up to 80-fold variability in IC_{50} values to nifurtimox. Therefore, it could be inferred that the increase in drug concentrations in the Post-Tt isolates have affected, in a certain way, the phenotypic, physiological or genetic characteristics of the original parasitic populations, but so far we do not have the experimental confirmation for this claim.

However, in the long-term, we can't exclude that the nifurtimox treatment may select for even more resistant phenotypes, as has been reported by Veloso *et al*[30] who induced benznidazole resistance to isolates obtained from dogs that have not received treatment for a long time. These resistant parasites were able to retain the resistant phenotype up to six months in the absence of the drug[31]. This result with Benznidazole could be extrapolated to ours if we take into consideration that Wilkinson *et al*[32] have detected cross-resistance to both the drugs.

As an exception for this tendency Post-Tt isolate 843.1, displayed increased susceptibility to nifurtimox. Although this behavior is difficult to explain, we believe that this particular isolate was present in the original in-

oculum and managed to avoid the host immune system by having higher virulence and/or a particular histotropism for tissues where an effective concentration of the drug was not possible to be achieved. Along these lines Martins[33] found that when mice were inoculated with a mixture of Benznidazole susceptible and resistant isolates, they were able to recover one more susceptible isolate after Benznidazole treatment. However, the authors did not provide an explanation for this apparent lack of selection process. The prior knowledge of IC_{50} values of the three Pre-Tt patients (22.35 ± 2.87 to 99.31 ± 4.57μM) may have helped us to suspect a nifurtimox therapeutic failure in these patients[34–35]. However, in opposition to this idea, Moreno *et al*[36] reported their failure to predict benznidazole treatment efficacy through the use of IC_{50} values.

CONCLUSION

Given the great heterogeneity in nifurtimox IC_{50} values in the isolates and clones from the OChD patients, we can infer that the therapeutic failure to nifurtimox could be due in part to a phenotypic variability that existed in the wild parasite population of the original source of contamination, which may or may not have a genetic basis. Although this aspect requires more experimental evidence, in future, the study of the pharmacological behavior of *T. cruzi* infective isolates may allow a differential and more suitable treatment regimen for OChD patients.

Conflict of interest

The authors declare no competing interests.

ACKNOWLEDGEMENTS

The Financial support for the study was provided by the Sección de Inmunología, Instituto de Medicina Tropical, Facultad de Medicina, Universidad Central de Venezuela (IMT-FM-UCV) and the Biotechnology Center of the Fundación Instituto de Estudios Avanzados (IDEA), Caracas-Venezuela.

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Correspondence to: Mr Arturo Muñoz Calderón, Sección de Inmunología, Instituto de Medicina Tropical, Facultad de Medicina, Universidad Central de Venezuela (IMT-FM-UCV), Zip–1041, Caracas, Venezuela.
E-mail: arturomc35@gmail.com

Received: 31 March 2018 *Accepted in revised form:* 19 July 2019