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The characterization of anti-*T. cruzi* activity relationships between ferrocenyl, cyrhetrenyl complexes and ROS release

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Abstract *Trypanosoma cruzi* (*T. cruzi*) is the parasite that causes Chagas disease. Nifurtimox is the most used drug against the *T. cruzi*, this drug increases intermediaries nitro group, being mainly responsible for the high toxicity component, for this reason it is important to study new organic compounds and thus improve therapeutic strategies against Chagas disease. The electronic effects of ferrocenyl and cyrhetrenyl fragments were investigated by DFT calculation. A close correlation was found between HOMO–LUMO gap of nitro radical NO_2^- with the experimental reduction potential found for nitro group and IC_{50} of two forms the *T. cruzi* (epimastigote and trypomastigote). The IC_{50} on human hepatoma cells is higher for both compounds compared to IC_{50} demonstrated in the two forms the *T. cruzi*, and additionally show reactive oxygen species release. The information obtained in this paper could generate two new drugs with anti-*T. cruzi* activity, but additional studies are needed.

Keywords *Trypanosoma cruzi* · Reactive oxygen species · Anti-chagasic · DFT

Abbreviations

ROS	Reactive oxygen species
T. cruzi	*Trypanosoma cruzi*

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HepG2 Human hepatoma cells
NFX Nifurtimox

Chagas disease, or trypanosomiasis in the United States, develops from the protozoa *Trypanosoma cruzi* (*T. cruzi*). It is one of the most common endemic diseases in Central and South America. The World Health Organization (WHO) estimates that approximately 6–7 million people in Latin America are infected or are serologically positive for *T. cruzi*. Up to 30 % of chronically infected people develop cardiac alterations and up to 10 % develop digestive, neurological or mixed alterations which may require specific treatment (WHO 2015). Trypanocidal drugs, such as Nifurtimox (NFX) (a nitrofurane-containing compound) and benznidazole (a nitroimidazol derivative), have been extensively used. These medications are considered less effective during the chronic phase of infection, and there is disagreement among scientists regarding their efficacy during this time period. Unfortunately, a major drawback of these active compounds is their high rates of adverse effects (Boiani et al. 2010; Maya et al. 2010).

Recently, a considerable amount of research has focused on understanding the mechanistic nature of this class of molecules. Most studies agree that the release of toxic intermediates that are formed by the one electron bioreduction of the nitro group is responsible for the high activity of these drugs (Maya et al. 2010; Arancibia et al. 2011; Hasslocher-Moreno et al. 2012; Miller et al. 2015).

The challenge of controlling and eventually eradicating Chagas disease requires the development of new drugs. In this regard, several organic compounds that contain 5-nitrofurane systems have been reported as potential antichagasic agents. Metal complexes also appear to be a promising alternative for the treatment of trypanosomiasis. Based on the results obtained previously for metal-based complexes using the 5-nitrofuryl-containing thiosemicarbazones ligand, proposed for the first time organometallic Rhenium compounds inspired in the anti-*T. cruzi* group 5-nitrofuryl, covantly attached to cyrhetrenyl or ferrocenyl fragments. Although the resulting derivatives were less active against epimastigotes of *T. cruzi* than NFX, the cyrhetrenyl complex, was more efficient as antichagasic agent compared with its ferrocenyl analogue or purely organic analogues. This suggests that the electron-withdrawing and bulky carbonyl ligands stabilize a large electron density on the metal in comparison to the ferrocenyl group. On the other hand, the presence of the organometallic moieties dramatically enhanced the cytotoxicity, probably by increasing the lipophilic character or by a possible synergism between the organometallic and 5-nitrofurfuryl groups (Arancibia et al. 2011, 2013).

More recently, we published four new bio-organometallics based on a 5-nitrothiophene pharmacophore and ferrocenyl and cyrhetrenyl Schiff bases. Only one compound presented better bioactivity than the furan analogues, and as reported in the previous study, the cyrhetrenyl compounds showed better activity against *T. cruzi* than the ferrocenyls, with IC_{50} values assayed on epimastigote and trypomastigote better than those exhibited by NFX. This evidence indicated that electronic effects of the organometallic fragments have a preponderant role in the anti-*T. cruzi* activity. In general, compounds possessing electronic communication between the organometallic moiety and the 5-nitroheterocyclic ring, such as ferrocenyl and cyrhetrenyl derivatives, show anti-chagasic activity. However, the presence of the electron-withdrawing cyrhetrenyl group, resulted in more active compounds in comparison to their ferrocenic analogues, indicating that electron-withdrawing effects are more efficiently transferred to the furane or thiophene groups facilitating the reduction of NO_2 to NO_2^- (Arancibia et al. 2013).

The electronic effects of anti-tubercular nitrofurans have been studied using theoretical calculations. This antitubercular activity was associated with a localized negative potential region near both oxygen atoms of the nitro group, which is extended to a lateral conjugated side arm (Zhou et al. 2013). Reduction of the nitro group of NFX produces a cycle of nitro anion radical production and a subsequent oxygen redox reaction, thereby yielding reactive oxygen species [(ROS) superoxide anion and H_2O_2]. These ROS have been proposed to explain the toxic effects against *T. cruzi* (Docampo and Moreno 1986). Researchers have shown that both antioxidant enzymes and ROS will increase in different growth phases of *T. cruzi* (Piacenza et al. 2009; Peloso et al. 2012). Therefore, ROS were shown to have an important role in programmed cell death in *T. cruzi* (Piacenza et al. 2007).

We sought to understand the stereoelectronic properties that govern the anti-*T. cruzi* activity of nitroheterocyclic organonometallic compounds. In this paper, we report a theoretical study that correlates the opposing electronic effects of ferrocenyl and cyrhetrenyl fragments with the reduction potential of the NO_2 group of the two nitrofurane derivatives, which is related to an increase in ROS levels. Furthermore, we report the characterization of structural anti-*T. cruzi* activity relationships between ferrocenyl and cyrhetrenyl complexes and increased ROS levels in human hepatoma cells (HepG2).

Several reports have indicated a correlation between trypanocidal activity and both the structural parameters and redox potential of the active compounds, using 5-nitrofurane (Porcal et al. 2007; Nogueira Silva et al. 2008). Thus, we sought to conduct a theoretical investigation regarding the properties of the organometallic-nitrofurane compounds. In doing so, we sought to understand the mechanism of action of these hybrid structures against the protozoan *T. cruzi*. (Fig. 1).

The molecular properties determined in this study were HOMO energy, GAP [ELUMO-EHOMO], and molecular orbitals. Table 1 summarizes the main electronic properties and in vitro activity of the compounds **1**, **2** and NFX. The calculations were performed for both the neutral and radical species. The electrochemical reductions occur in the nitro group because it is the most susceptible moiety of compounds **1** and **2** to nucleophilic attack. These data are also in agreement with the charges and molecular orbital calculations, thereby indicated that the NO_2 moiety is more electrophilic and that the LUMO is localized in the NO_2 group (Fig. 2). Furthermore, according to GAP and HOMO energies that are available to reduce compounds, the stability to reduce species is also in agreement with the experimental data for in vitro trypanocidal activity on trypomastigotes and in vitro antriroliferative activity on epimastigotes (Table 1). The difference of energy between the frontier orbitals was hypothesized to be associated with the trypanocidal activity in the nitrofurane-containing ruthenium complexes due to the easy generation of the nitro radical (Nogueira Silva et al. 2008).

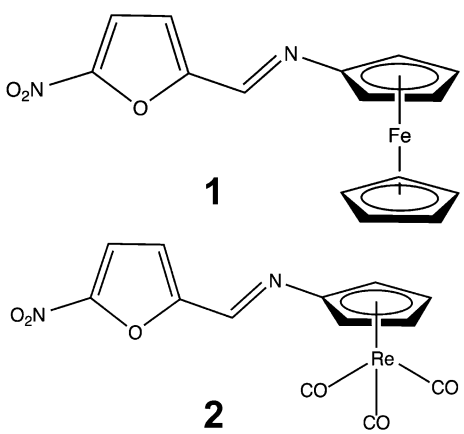

Fig. 1 Ferrocenyl and cyrhetrenyl complexes. **1** (Nitro-2-furfurylideneamino)ferrocene. **2** (5-Nitro-2-furfurylideneamino)cyrhetrene

Thus, the best energies of compounds **1** and **2** could be a consequence of a higher stability of the reduced complexes and an accessibility of the nitro group to reduction (Table 1). Compounds that possess electronic communication between the organometallic and 5-nitofurane groups, such as the ferro, ferrocenyl, and cyrhetrenyl derivatives, demonstrate an enhanced antichagasic activity (Dias et al. 2007; Nogueira Silva et al. 2008). This result is likely due to its electron-withdrawing effects that are efficiently transferred to the furane group, thereby facilitating reductions of NO_2 to NO_2^-. The calculations indicate that synergism exists between the organometallic and 5-nitrofurfuryl groups. For this reason, the electronic effects should be considered in designing new molecules with potential anti-*T. cruzi* activity. However it is also important to study the effect of drugs on human cells.

The liver is one of the most important organs, particularly in the detoxification and removal of many toxic chemicals. It is often also the target organ of various toxins or drugs (Pugh et al. 2009). NFX treatment increased biliary secretion and the loss of hepatic glutathione in the rat liver, but NFX was completely metabolized after 2–4 h and did not causing damage to liver cells (Dubin et al. 1983; González-Martin et al. 1993). Moreover, it has been used in rat liver microsomes to assess the oxidative stress that is induced by NFX drug biotransformation (Letelier et al. 2004). For these reasons, we used human hepatoma cells (HepG2) to assess anti-*T. cruzi* activity.

The cytotoxicity of compounds **1** and **2** was assessed using an MTT assay. HepG2 cells experienced a decrease in viability at the highest compound concentrations that were tested. The estimated IC_{50}

Table 1 Comparison of in vitro activity and molecular properties for ferrocenyl (**1**) and cyrhetrenyl (**2**) complexes and NFX

	ROS	ω	IC_{50} (epim)	IC_{50} (tryp)	$E_{1/2}(NO_2$ group)	HOMO (eV)	HOMO–LUMO GAP (eV)
1	2.5	−23.8	48.8 ± 0.8[b]	12.3 ± 1.7[b]	−0.735	−4.61 [−4.18][a]	0.89 [1.22][a]
2	4.2	−41.4	12.7 ± 3.1[b]	0.4 ± 0.1[b]	−0.630	−4.98 [−5.25][a]	1.04 [2.38][a]
NFX	2.8	−81.8	17.2 ± 3.3[c]	17.2 ± 3.3[c]	−0.880	−5.72 [−3.19][a]	1.73 [1.10][a]

The IC_{50} values are shown as the mean ± SD (μM) from three separate experiments

NFX Nifurtimox, *epim* epimastigote, *tryp* trypomastigote

[a] Reduces compound

[b] Arancibia et al. (2011)

[c] Arancibia et al. (2013)

[d] Letelier et al. (2004)

values (concentration at which death occurred in 50 % of HepG2 cells) of compounds **1** (Fig. 3a, c, e) and **2** (Fig. 3b, d, f) were tested at 24 (Fig. 3a, b), 48 (Fig. 3c, d) and 72 h (Fig. 3e, f). HepG2 cells experienced an IC_{50} of 103.7 ± 9.8 μM for compound **1**. However, this value was not determined in compound **2** at 24 h (for experimental reasons). The IC_{50} was 68.2 ± 26.1 μM for compound **1** and 62.7 ± 6.9 μM for compound **2** at 48 h, and the IC_{50} was 68.3 ± 32.2 μM for compound **1** and 50.9 ± 3.2 μM at 72 h. The IC_{50} of compounds **1** and **2** in HepG2 cells was higher than the IC_{50} of the compounds against epimastigoste and trypamastigote that are shown in Table 1. These results demonstrate that these drugs exert their cytotoxic effect on the *T. cruzi* without harming the host cells.

The principal activity/toxicity of NFX in *T. curzi* is due to its ability to form free radicals that react with molecular oxygen to form ROS, such as the superoxide anion, hydrogen peroxide, and hydroxyl radicals (Docampo et al. 1981; Nozaki et al. 1996). For this reason, we conducted ROS production assays in the HepG2 cells for compounds **1** and **2**. ROS was monitored with redox-sensitive dyes. 2′,7′Dichlorofluorescein (DCF) was used for the detection of hydrogen peroxide and nitric oxide (Carter et al. 1994). As shown in Fig. 4, exposure of HepG2 cells to a dose of compound **1** (50 and 100 μM) resulted in the significant overproduction of ROS levels at 24, 48 and 72 h (Fig. 4a–c). Only compound **1** (10 μM) at 24 h produced significant differences (Fig. 4a). Compound **2** also demonstrated significant differences but only at 72 h for all concentrations (10, 50 and 100 μM). At this time, ROS levels were increased by approximately four times There were also significant differences between compounds **1** and **2** only at 72 h at all concentrations (Fig. 4c). These data were also confirmed by the observation of increases in intracellular ROS when HepG2 cells were treated (24, 48 and 72 h) with both anti-*T. cruzi* compounds at a concentration of 50 μM (4D). These results demonstrated that alterations in the redox potential of the host cells could generate a cytotoxic effect on *T. cruzi*.

In recent years, there have been significant efforts to improve anti-*T. cruzi* activity drugs beyond the identification of their mechanisms of action and their effects on host cells. Understanding the mode of action of the compounds and their structure allows for the possibility of modifications to these structures, which would allow for the further improvement in their mechanism of action and effectiveness against *T. cruzi*. Our results demonstrate that compound **2** has a greater effect on ROS production at low concentrations. These concentrations do not affect the viability of the host cells. We hypothesized that the modification of the script of NFX compounds that increases the redox potential of the host cell increases intracellular ROS generation. This increase in ROS could damage *T. cruzi*, thereby rendering it ineffective.

We then sought to determine the ability of the compounds to increase ROS levels without altering host cell viability. Although the increases in ROS are toxic to *T. cruzi*, they can also damage DNA, proteins and lipids of the host cells. It is known that redox potential alterations of HepG2 apoptotic cells cause cell death in this cell line and in other tumor lines (Manov et al. 2004; Circu and Aw 2010). The two main drugs that are clinically used to treat *T. cruzi* (NFX and Benznidazole) are hypothesized to induce

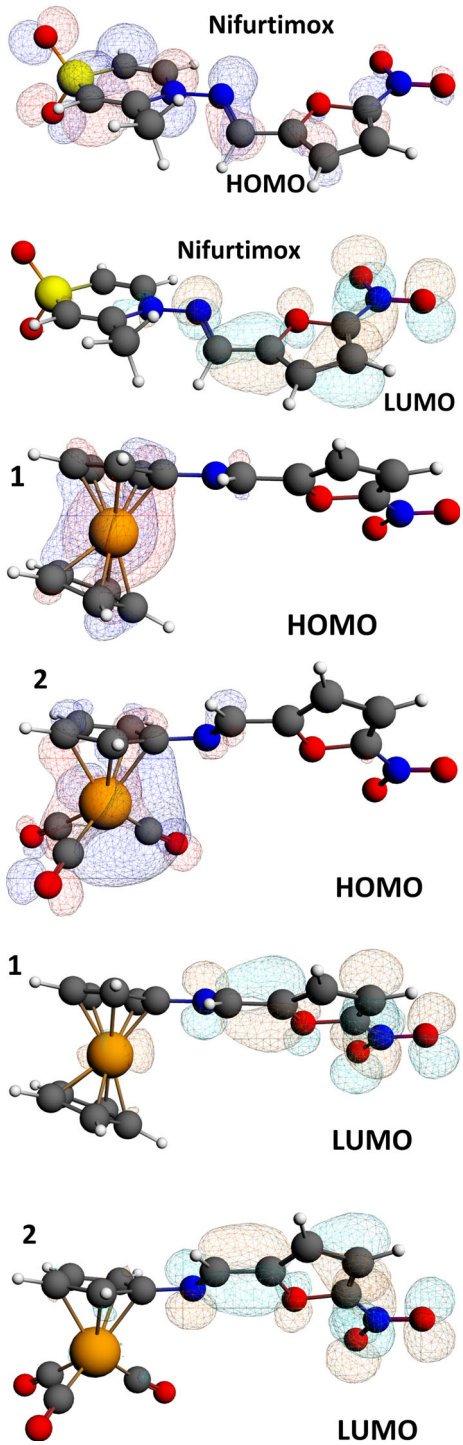

Fig. 2 Molecular orbitals of compounds **1**, **2** and NFX

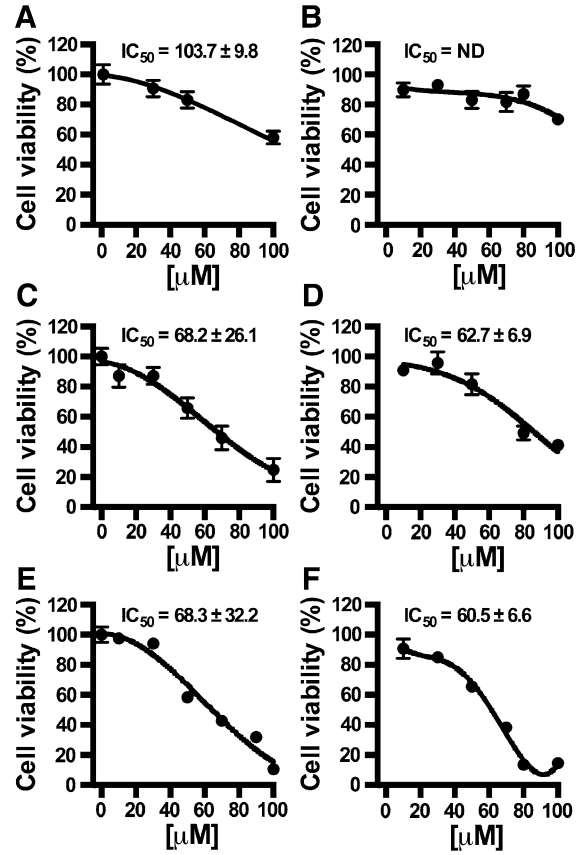

Fig. 3 Effect of the compounds **1** and **2** on the growth of HepG2. The cells were treated with various concentrations of the compounds **1** (**a**, **c**, **e**) and **2** (**b**, **d**, **f**) for 24 h (**a**, **b**), 48 h (**c**, **d**) and 72 h (**e**, **f**). Cell viability was measured using the MTT assay. The *inset* contains IC_{50} values ± SD. *ND* no determined

the formation of ROS in mammalian tissues (Docampo et al. 1981; Maya et al. 2007). Furthermore, for 30 years, scientists have known that *T. cruzi* is highly susceptible to intracellular environments with high levels of ROS, mainly due to the absence of catalase activity (Docampo 1990). Both anti-*T. cruzi* compounds increase ROS production (Fig. 4) at lower concentration for the IC_{50} observed in HepG2 cells (Fig. 3).

As expected, the compounds **1** and **2** induce ROS in HepG2 cells, increased the levels of ROS in potential host cells, and consequently would generate a toxic microenvironment that could affect the proliferation of *T. cruzi* without harming host cells.

In conclusions, the charge density, HOMO or GAP of the radical species demonstrate that the electronic effects of the ferrocenyl and cyrhetrenyl fragments of imine derivatives influence their trypanosomal activity and their reduction potential of the nitro substituent. This indicates that electron-withdrawing

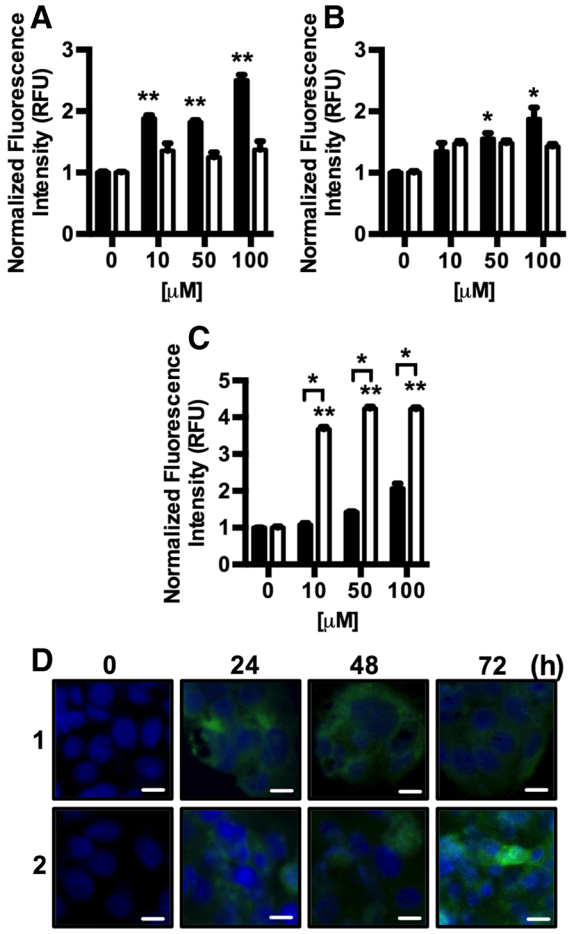

Fig. 4 Effects of the compounds **1** and **2** on intracellular ROS generation in HepG2 cells. Cells were treated with **1** (*Black bars*) and 2 (*white bars*) for 24 h (**a**), 48 h (**b**) and 72 h (**c**). **d** Cells were treated with **1** and **2** 100 μM for 24, 48 and 72 h, the photomicrographs shown here are from one representative experiment that was repeated three times with similar results. *P < 0.01, **P < 0.001, indicate statistically significant differences. Nuclei were stained using DAPI. *Bars* 20 μm

effects of the cyrhetrenyl group are efficiently transferred to the furane group, thereby facilitating reductions of NO_2 to NO_2^-. Furthermore, we observed a close correlation between the HOMO–LUMO gap of nitro radical NO_2^- and the experimental reduction potential that was observed for the nitro group and the IC_{50} of the two forms on *T. cruzi* (epimastigote and trypamostigote). The IC_{50} values obtained from the human cells that were stimulated with compounds **1** and **2** are higher in than those obtained when these compounds were studied with *T. cruzi* (Arancibia et al. 2011, 2013). This finding establishes that the use of these anti-*T. cruzi* agents at lower concentrations does not produce death or alteration of human cells. Moreover, these compounds are able to increase the capacity to generate ROS. This effect has also been observed in other anti-*T. cruzi* agents (Boiani et al. 2010; Paes et al. 2011; Peloso et al. 2012). Both compounds **1** and **2** could generate a direct adverse effect on *T. cruzi* through increased intracellular ROS. These observations could improve the mechanism of action of the compounds **1** and **2** for their use as a therapy against *T. cruzi*.

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