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Manganese(II) complexes with N^4-methyl-4-nitrobenzaldehyde, N^4-methyl-4-nitroacetofenone, and N^4-methyl-4-nitrobenzophenone thiosemicarbazone: Investigation of *in vitro* activity against *Trypanosoma cruzi*

Denise da Gama Jaén Batista [a], Patrícia Bernardino da Silva [a], Daniela R. Lachter [b], Renata S. Silva [b], Ricardo Q. Aucelio [b], Sonia R.W. Louro [c], Heloisa Beraldo [d], Maria de Nazaré C. Soeiro [a,*], Letícia R. Teixeira [d]

[a] *Laboratório de Biologia Celular, Instituto Oswaldo Cruz, FIOCRUZ, Rio de Janeiro (RJ), Brazil*
[b] *Departamento de Química, Pontifícia Universidade Católica do Rio de Janeiro, 22653-900 Rio de Janeiro (RJ), Brazil*
[c] *Departamento de Física, Pontifícia Universidade Católica do Rio de Janeiro, 22653-900 Rio de Janeiro (RJ), Brazil*
[d] *Departamento de Química, Universidade Federal de Minas Gerais, 31270-901 Belo Horizonte (MG), Brazil*

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ABSTRACT

Thiosemicarbazones are known to be active against different pathogenic microorganisms including *Trypanosoma cruzi*, the etiological agent of Chagas disease. In the search for new therapeutic drugs against this illness, the complexes $[Mn(H4NO_2Fo4M)_2Cl_2]$ (**1**), $[Mn(H4NO_2Ac4M)_2Cl_2]$ (**2**) and $[Mn(H4NO_2Bz4M)_2Cl_2]$ (**3**) of N^4-methyl-4-nitrobenzaldehyde thiosemicarbazone ($H4NO_2Fo4M$), N^4-methyl-4-nitroacetophenone thiosemicarbazone ($H4NO_2Ac4M$) and N^4-methyl-4-nitrobenzophenone thiosemicarbazone ($H4NO_2Bz4M$) were obtained and screened *in vitro* against bloodstream and intracellular forms of *T. cruzi*. $H4NO_2Fo4M$, $H4NO_2Ac4M$ and their Mn(II) complexes displayed poor effect on bloodstream trypomastigotes, with IC_{50} values ranging from 68 to >200 μM. However, although $H4NO_2Bz4M$ was also not active, its corresponding Mn(II) complex presented high effect on this *T. cruzi* form, with an IC_{50} value of 19 μM. The effect of complex (**3**), against trypomastigotes of *T. cruzi* supports further *in vitro* as well as *in vivo* studies.

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1. Introduction

Chagas' disease is a tropical neglected illness that is the leading cause of heart disease in Latin America where it affects approximately 12 million people living in very poor social conditions [1]. In addition, the globalization of Chagas' disease is now acknowledged as it can also be found throughout the world due to international immigration [2,3]. Thus, this anthropozoonosis, discovered a century ago by the Brazilian physician Carlos Chagas, still represents a serious Public Health problem in the affected areas claiming for care and resolution of its current challenges, including the imperative need to sustain public policies related to the transmission control and the urgent requirement for new chemotherapic agents [4,5]. In fact, since the existing therapy, based on the nitroheterocycles nifurtimox (3-methyl-4-[(5-nitrofurfurylidene) amino]thiomorpholine-1,1-dioxide) and benznidazole (2-nitro-*N*-(phenylmethyl)-1*H*-imidazole-1-acetamide), developed empirically over three decades ago leads to severe side effects, requires long term treatment and is of limited efficacy especially in its chronic phase, identification of new drugs for Chagas disease is indisputable [6–8].

Thiosemicarbazones and their metal complexes represent an interesting class of compounds with a wide range of pharmacological applications [9]. Many examples of this class of small molecules have been evaluated over the last 50 years as having antitumor [10–12], antibacterial [13,14], antifungal [13], antiviral [15–17] and antiprotozoal activities [18,19]. Additionally, some studies demonstrated that novel thiosemicarbazones exhibit significant *in vitro* activity against trypanosomatids such as *Leishmania* sp. [20], *Trypanosoma brucei* [19], and *Trypanosoma cruzi* [21–23].

Some of us recently started an investigation on the pharmacological profile of 4-nitroacetophenone-derived thiosemicarbazones and their metal complexes. It has been shown that these thiosemicarbazones and their Cu(II) complexes present significant *in vitro* anti-trypanosomal activity, the complexes resulting to be at least 5 times more active than the free ligands [18]. Recently, Ru(II) complexes with N^4-methyl-4-nitrobenzaldehyde thiosemicarbazone ($H4NO_2Fo4M$) and N^4-methyl-4-nitroacetophenone thiosemicarbazone ($H4NO_2Ac4M$) containing 1,4-bis(diphenylphospine) butane (dppb) and 2,2′-bipyridine (bipy), 4,4′-dimethyl-2,2′-bipyridine (Mebipy) or 1,10-phenanthroline (phen) as co-ligands have

* Corresponding author. Tel.: +55 21 25984534; fax: +55 21 2562 1368.
E-mail address: soeiro@ioc.fiocruz.br (Maria de Nazaré C. Soeiro).

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been developed [24]. All complexes revealed to be at least 10 times more active than their corresponding free thiosemicarbazones [24].

In the present study, Mn(II) complexes with N^4-methyl-4-nitrobenzaldehyde thiosemicarbazone ($H4NO_2Fo4M$), N^4-methyl-4-nitroacetophenone thiosemicarbazone ($H4NO_2Ac4M$) and N^4-methyl-4-nitrobenzophenone thiosemicarbazone ($H4NO_2Bz4M$) (Fig. 1) were obtained and their cytotoxicity, trypanocidal efficacy and selectivity were evaluated *in vitro* against both bloodstream trypomastigotes and intracellular amastigotes of *T. cruzi*.

2. Experimental

2.1. Physical measurements

Elemental analyses were performed on a Fison equipment, model EA 1108. A Radiometer Copenhagen Meter Lab., model CDM 230 was employed for molar conductivity measurements. Infrared spectra (KBr pellets) were obtained using a BOMEM MICHELSON instrument, model 102. Magnetic susceptibility measurements were carried out on a Johnson Matthey MSB/AUTO balance. X-band electron paramagnetic resonance (EPR) spectra were obtained with a Bruker ESP300E equipment with modulation frequency of 100 kHz and modulation amplitude of 0.1 or 1 mT. Samples in the solid state were measured in glass capillaries at room temperature; frozen ethanol solutions were measured in Teflon® tubes of 3 mm of internal diameter immersed in liquid N_2 (77 K).

The electrochemical experiments were carried out at room temperature in dichloromethane containing 0.1 mol L^{-1} tetrabutylammonium perchlorate (TBAP) (Fluka Purum) using an electrochemical analyzer from Bioanalytical Systems Inc. (BAS), model 100BW. The working and auxiliary electrodes were stationary Pt foils, and the reference electrode was Ag/AgCl, a medium in which ferrocene is oxidized at 0.48 V (Fc^+/Fc).

Luminescence studies were made on a Perkin–Elmer LS 55 spectrofluorimeter using 10 nm spectral bandpass. Fluorescence measurements were performed in 1 cm quartz cells using solutions prepared either in CH_2Cl_2 or in dimethylformamide (DMF). In solid state, fluorescence was made using a front solid surface apparatus (Perkin–Elmer) and a cell with a quartz window. For phosphorescence measurements, the front solid surface apparatus was also employed and the signals were measured directly from the surface of dry low background cellulose substrates [25] using both detector delay and detector gate times set at 3 ms. Measurements were made in an oxygen free environment established by the use of dried nitrogen gas. Solutions (5 μL) of the free thiosemicarbazones and their metal complexes, prepared in CH_2Cl_2, were deposited on the center of the cellulose substrates. These substrates were previously spotted with 5 μL of heavy atom salt solutions – $Pb(NO_3)_2$, $CdCl_2$, $TlNO_3$, $AgNO_3$, $HgCl_2$ – which were used as potential phosphorescence inducers. Measurements were made after the substrates were dried under vacuum (2 h).

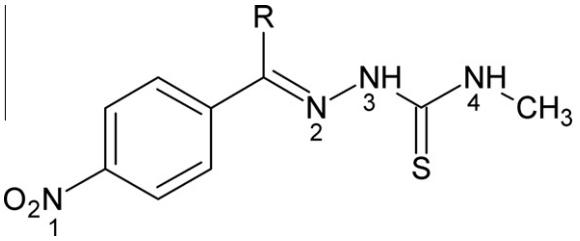

Fig. 1. Structures of N^4-methyl-4-nitrobenzaldehyde thiosemicarbazone (R = H, $H4NO_2Fo4M$), N^4-methyl-4-nitroacetophenone thiosemicarbazone (R = CH_3, $H4NO_2Ac4M$) and N^4-methyl-4-nitrobenzophenone thiosemicarbazone (R = C_6H_5, $H4NO_2Bz4M$).

2.2. Drugs

N^4-methyl-4-nitrobenzaldehyde thiosemicarbazone ($H4NO_2Fo4M$), N^4-methyl 4-nitroacetophenone thiosemicarbazone ($H4NO_2Ac4M$) and N^4-methyl-4-nitrobenzophenone thiosemicarbazone ($H4NO_2Bz4M$) were prepared as described in the literature [18,26]. Briefly, equimolar amounts of 4-nitrobenzaldehyde, 4-nitroacetophenone and 4-nitrobenzophenone (6.6×10^{-3} mol) were mixed with N^4-methyl thiosemicarbazide in absolute ethanol (40 mL) with addition of 2–4 drops of concentrated sulfuric acid as catalyst. The reaction mixture was kept under reflux for 6–7 h. The precipitated material were filtered, washed with diethylether and dried under vacuum.

The complexes were obtained by dissolving the desired thiosemicarbazone (0.40 mmol) in ethanol (20 mL) with gentle heating and stirring. After cooling, the solution to room temperature, $MnCl_2{\cdot}4H_2O$ (0.20 mmol) was added. The reaction was stirred at room temperature for 24 h. The solids which precipitated were filtered and washed with diethyl ether and dried.

2.2.1. $[Mn(H4NO_2Fo4M)_2Cl_2]$ (**1**)

Orange solid; yield: 78%. *Anal.* Calc. ($C_{18}H_{20}Cl_2MnN_8O_4S_2$): C, 35.89; H, 3.35; N, 18.60. Found: C, 35.79; H, 3.33; N, 18.58. IR (KBr, cm^{-1}): ν(C=C) + ν(C=N) 1561, $\nu_{ass}NO_2$ 1509, ν_sNO_2 1335, ν(C=S) 824. Molar conductivity (1×10^{-3} mol L^{-1} acetone): 1.70 μS cm^{-1}. μ_{eff}: 1.89 BM.

2.2.2. $[Mn(H4NO_2Ac4M)_2Cl_2]$ (**2**)

Yellow solid; yield: 72%. *Anal.* Calc. ($C_{20}H_{24}Cl_2MnN_8O_4S_2$): C, 38.10; H, 3.84; N, 17.77. Found: C, 38.10; H, 3.83; N, 17.76%. IR (KBr, cm^{-1}): ν(C=C) + ν(C=N) 1547, $\nu_{ass}NO_2$ 1507, ν_sNO_2 1339, ν(C=S) 847. Molar conductivity (1×10^{-3} mol L^{-1} acetone): 3.41 μS cm^{-1}. μ_{eff}: 1.86 BM.

2.2.3. $[Mn(H4NO_2Bz4M)_2Cl_2]$ (**3**)

Orange solid; yield: 88%. *Anal.* Calc. ($C_{30}H_{28}Cl_2MnN_8O_4S_2$): C, 47.75; H, 3.74; N, 14.85. Found: C, 47.69; H, 3.72; N, 14.81%. IR (KBr, cm^{-1}): ν(C=C) + ν(C=N) 1545, $\nu_{ass}NO_2$ 1513, ν_sNO_2 1338, ν(C=S) 825. Molar conductivity (1×10^{-3} mol L^{-1} acetone): 2.76 μS cm^{-1}. μ_{eff}: 1.97 BM.

Stock solutions of the thiosemicarbazones and their Mn(II) complexes (50 mM) were prepared in dimethylsufoxide (DMSO) and dimethylformamide (DMF). Fresh dilutions were prepared immediately before use, with the final solvent content never exceeding 0.4%, which led to no toxicity to the parasite or the mammalian host cells.

2.3. Parasites

Y strain of *T. cruzi* was used throughout the experiments. Bloodstream forms were harvested by heart puncture from *T. cruzi*-infected Swiss mice at the peak of parasitemia [27].

2.4. Mammalian cell cultures and toxicity assays

For both drug toxicity and infection assays, primary cultures of embryonic cardiomyocytes (CM) were obtained following the previously described method [28]. After purification, the CM were seeded at a density of 0.1×10^6 cells/well into 24-well culture plates, or 0.05×10^6 cell/well into 96-well microplates, containing gelatin-coated cover slips and sustained in Dulbecco's modified medium supplemented with 10% horse serum, 5% fetal bovine serum, 2.5 mM $CaCl_2$, 1 mM L-glutamine and 2% chicken embryo

extract (DMEM). All the cultures were maintained at 37 °C in an atmosphere of 5% CO_2, and the assays were run at least three times in duplicate.

In order to rule out toxic effects of the compounds on host cells, uninfected CM were incubated for 24 and 72 h at 37 °C in presence or absence of the compounds (up to 200 μM) diluted in DMEM. Then their morphology was evaluated by light microscopy and loss of cellular viability measured by the MTT colorimetric assay [29]. The absorbance was measured at 490 nm with a spectrophotometer (VERSAmax tunable, Molecular Devices, USA) allowing the determination of LC_{50} values (drug concentration that reduces 50% of cellular viability) and the respective selective indexes ($SI = LC_{50}/IC_{50}$).

2.5. Trypanocidal analysis

For the *in vitro* analysis on bloodstream trypomastigotes, the parasites were incubated at 37 °C for 24 h in the presence of increasing doses (0–200 μM) of each compound diluted in Dulbecco's modified medium supplemented with 5% fetal bovine serum and 1 mM L-glutamine (DMES) [30]. The incubation of parasites with benznidazole (0–125 μM) or with the Mn(II) salt (0–400 μM) alone, under the same conditions, was also performed. After drug incubation, death rates were determined by light microscopy through the direct quantification of live parasites using a Neubauer chamber, and the IC_{50} values (drug concentration that reduces 50% of the number of the parasites) were then calculated [30].

For the analysis of intracellular parasites, after the initial 24 h of host cell-parasite contact (using a CM: trypomastigotes ratio of 1:10), the cultures were washed to remove free parasites followed by the treatment with the compounds (0.2 mL of the drugs, at crescent concentrations up to 200 μM). Infected and untreated CM were used as controls. All cultures were maintained at 37 °C in an atmosphere of 5% CO_2 and air, and the medium, with or without the drugs, was replaced everyday. After 72 h of drug exposure, supernatants of untreated and treated cultures were recovered, the number of released parasites quantified by light microscopy using a Neubauer chamber [31,32], and IC_{50} values calculated.

All procedures were carried out in accordance with guidelines established by FIOCRUZ Committee of Ethics for the Use of Animals (approved protocol number L08/09).

3. Results and discussion

3.1. Microanalyses, molar conductivity and magnetic susceptibility studies

Microanalyses suggest the formation of $[Mn(H4NO_2Fo4M)_2Cl_2]$ (**1**), $[Mn(H4NO_2Ac4M)_2Cl_2]$ (**2**), $[Mn(H4NO_2Bz4M)_2Cl_2]$ (**3**) in which the thiosemicarbazones coordinate as neutral ligands. The molar conductivity data reveal that the complexes are non-electrolytes, in accordance with the proposed formulations. The values of magnetic moments, in the 1.86–1.97 BM range, are close to the calculated value of 1.73 BM, characteristic of the presence of one unpaired electron as in low spin Mn(II) complexes.

3.2. Infrared spectral studies

The ν(C=C) + ν(C=N) composed mode observed at 1583–1590 cm^{-1} in the spectra of the thiosemicarbazones shifts to 1545–1561 cm^{-1} in the spectra of the complexes, indicating coordination of the azomethine nitrogen N(2) [18,26].

The ν(C=S) absorption at 824–832 cm^{-1} in the spectra of the uncomplexed thiosemicarbazones is observed at 825–847 cm^{-1} in those of the complexes, in accordance with coordination through a thione sulfur [33–35]. The 7–19 cm^{-1} shift observed upon complexation is compatible with coordination of a neutral thiosemicarbazone in all cases [33–35]. Hence the infrared spectra indicate coordination through the N–S chelating system.

3.3. EPR spectral studies

The X-band EPR spectra of complexes (**1**)–(**3**) obtained at room temperature for the solid state samples, and at 77 K for the ethanol solutions are presented in Fig. 2. The EPR spectra of the powder samples show a broad signal with *g* value close to 2.01. In polycrystalline samples at room temperature Mn(II) complexes give very broad signals, which may be due to dipolar interactions and enhanced spin lattice relaxation.

Mn(II) presents a $3d^5$ electronic structure. If the ligand-field splitting of the *d* orbitals is not too large the ions will present a high spin state, $S = 5/2$. For a large ligand-field splitting, spin pairing leads to the low spin state, $S = 1/2$. Even in the high spin case, the effective spin is usually 1/2, because the distorted ligand-field symmetry displaces the $m_S = \pm3/2$ and $\pm5/2$ levels to higher energy values and only transitions between the levels $m_S = \pm1/2$ are induced by the microwave. However, in this case, the effective g factor is very anisotropic. In the low spin case the g values are around the free-electron value ($g = 2.0023$) [36]. The ^{55}Mn nucleus presents a nuclear spin $I = 5/2$, so that the EPR spectrum of a Mn(II) solution at room temperature exhibits a characteristic six component hyperfine splitting. If the symmetry around Mn(II) is distorted due to complexation, the resonances become anisotropic and a randomly oriented sample may exhibit such a broad line that detection becomes difficult.

In the case of the solid state complexes (**1**)–(**3**), the EPR spectra are broad and isotropic (Fig. 2a). The hyperfine lines are not resolved. The *g* factor, 2.009, is very close to the free-electron value, suggesting a low spin Mn(II) ion, coming from a strong ligand-field. Complex (**3**), however, presented a much broader line (50 mT) than the others (20 mT). This is unexpected since the bulky ligand $H4NO_2Bz4M$ would weaken the magnetic dipolar interactions that broaden the lines. In general, dilution leads to line narrowing and better resolved spectra. In these complexes this does not occur, since both 1 and 0.1 mM ethanol frozen solutions presented the same 20 mT linewidth as the powder spectrum at room temperature (Fig. 2b). This suggests that line broadening was caused by the Mn(II)–ligand association with a low-symmetry ligand-field, not to the magnetic dipolar interaction between Mn(II) ions.

3.4. Electrochemical studies

The voltammograms of the thiosemicarbazones show a quasi-reversible process between −0.93 and −1.02 V, which has been attributed to the formation of the $Ar–NO_2^{\cdot-}$ radical. The second process between −1.11 and −1.34 V has been assigned to the formation of the Ar–NHOH species and the irreversible oxidation at 0.17–0.21 V to the formation of Ar–NO. The observed processes correspond to the well known mechanism for the nitro aromatic reduction, as previously proposed [18,26,37–40] (Fig. 3a).

The voltammograms of complexes (**1–3**) show all the events observed in the voltammograms of the free ligands (Fig. 3b). For complexes (**1**) and (**2**) the cyclic voltammograms show an irreversible process around −1.0 V attributed to the formation of the $Ar–NO_2^{\cdot-}$ radical. For complex (**3**), this process is quasi-reversible and the reduction with the formation of the $Ar–NO_2^{\cdot-}$ radical is observed at −1.00 V followed by its successive oxidation at −0.87 V. A second quasi-reversible reduction attributed to the formation of the Ar–NHOH species occurs between −1.26 and −1.22 V, followed by its respectively oxidation (−1.13 to −1.11), and an irreversible oxidation attributed to the formation of the Ar–NO species is

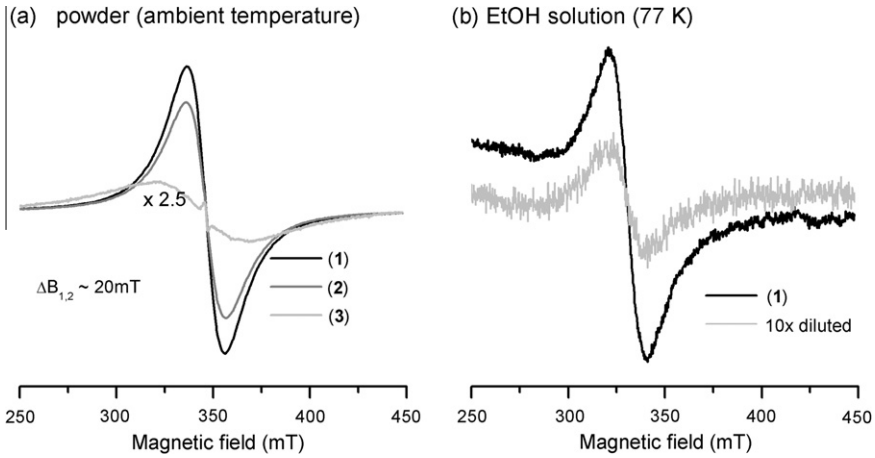

Fig. 2. (a) EPR spectra of complexes (**1**)–(**3**) in the solid state (powder samples) at room temperature. (b) EPR spectra of 1.0 and 0.1 mmol L^{-1} ethanol solutions of complex (**1**). Complexes (**2**) and (**3**) presented similar spectra.

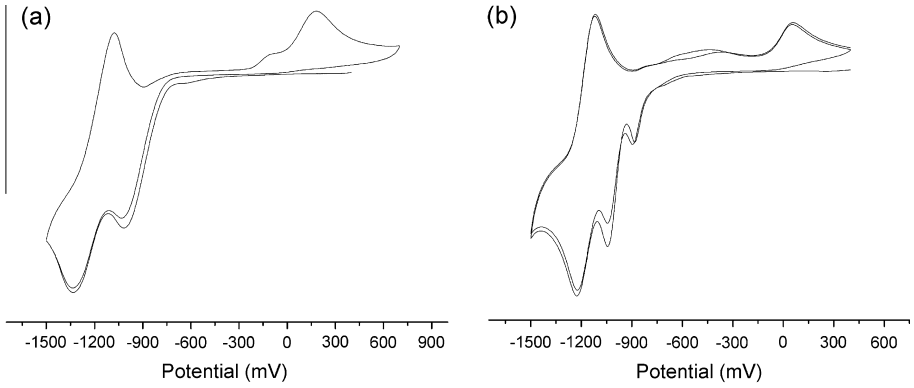

Fig. 3. Cyclic voltammograms of (a) $H4NO_2Fo4M$ and (b) $[Mn(H4NO_2Fo4M)_2Cl_2]$ (**1**) (CH_2Cl_2, 0.1 mol L^{-1} TBAP).

observed in the 0.48–0.83 V range for complexes (**1–3**). For all complexes the remaining processes are attributed to the irreversible reduction Mn(II)/Mn(I) (Table 1).

Experiments carried out on the main drugs used for the Chagas' disease treatment (nirfutimox and benznidazole) suggest that intracellular nitro-moiety reduction followed by redox cycling yielding reactive oxygen species may be their major mode of action against *T. cruzi* [22].

In the present work the quasi-reversible process between −1.00 and −1.08 V has been attributed to the formation of the $Ar–NO_2^{\cdot-}$ radical. The reported value for the same process in nirfutimox [22] falls into this same range. Interestingly, complexes (**1–3**) have adequate NO_2-reduction potential to act against *T. cruzi via* a redox cycling process.

3.5. Luminescence studies

Fluorescence studies in solution and in solid state as well as fosforescence using cellulose substrates have been measured for the N(4)-methyl nitrotiosemicarbazones and their Mn(II) complexes.

In the solid state, $H4NO_2Fo4M$, $H4NO_2Ac4M$ and $H4NO_2Bz4M$ present similar fluorescence spectra as indicated in Table 2. The maximum wavelength of excitation (λ_{exc}) and the maximum wavelength of emission (λ_{em}) appeared respectively at the 211–213 nm range and at the 372–373 nm range. Fluorescence from these compounds comes from the aromatic system; however, its low intensity can be explained by the presence of the NO_2 electron-withdrawing substituent that decreases the molecule fluorescence quantum yield by introducing a low lying n, π^* state. The long chain substitutions in the aromatic system also contribute with the decrease of fluorescence by favoring energy loss by radiationless processes.

The Mn(II) complexes present the characteristic fluorescence bands of the free thiossemicarbazones, indicating that metal coordination did not induce any relevant spectral changes. In the case of complex (**1**), a small fluorescence increasing after complexation has been observed. On the other hand, for complex (**3**) a decreasing

Table 1
Cyclic voltammetry data (V) for complexes (**1–3**) (0.100 V s^{-1}, CH_2Cl_2, 0.1 mol L^{-1}, TBAP).

Complex	$Ar–NO_2^{\cdot-}$		Ar–NHOH		Ar–NO	Mn^{II}/Mn^{I}
	E_{pc}	E_{pa}	E_{pc}	E_{pa}	E_{pa}	
$[Mn(H4NO_2Fo4M)_2Cl_2]$ (**1**)	−1.04	–	−1.22	−1.11	0.58	−0.89
$[Mn(H4NO_2Ac4M)_2Cl_2]$ (**2**)	−1.08	–	−1.25	−1.14	0.83	−0.91
$[Mn(H4NO_2Bz4M)_2Cl_2]$ (**3**)	−1.00	−0.87	−1.26	−1.13	0.48	−0.81

Table 2
Wavelengths of excitation (λ_{exc}, nm) and emission (λ_{em}, nm) and intensity of the fluorescence absorptions for the thiosemicarbazones and their Mn(II) complexes in the solid state.

Compound	λ_{exc} (nm)	λ_{em} (nm)	Intensity
$H4NO_2Fo4M$	211	373	102
$[Mn(H4NO_2Fo4M)_2Cl_2]$ (**1**)	211	374	147
$H4NO_2Ac4M$	213	373	115
$[Mn(H4NO_2Ac4M)_2Cl_2]$ (**2**)	213	374	111
$H4NO_2Bz4M$	212	372	188
$[Mn(H4NO_2Bz4M)_2Cl_2]$ (**3**)	212	373	109

of fluorescence was observed in comparison to the fluorescence from the free ligand. Finally, complex (**2**) and the free thiosemicarbazone presented identical spectra in terms of spectral position and signal intensity.

In CH_2Cl_2 solution, the $H4NO_2Fo4M$, $H4NO_2Ac4M$ and $H4NO_2Bz4M$ fluorescence spectra are very similar with λ_{exc} in the 214–216 nm range and λ_{em} in the 293–295 nm range. Comparative to the ones observed in solid state, the λ_{em} of the complexes in CH_2Cl_2 appeared in a significantly different spectral region (hypsochromic shift) indicating that solvation played an important role in excited state stabilization for these thiosemicarbazone and their complexes. In fact, when a more polar solvent (DMF) was employed, total fluorescence quenching was observed. Interaction between the withdrawing group (NO_2), which has great influence on the luminescence properties of these molecules, and the solvent (either polar of non-polar) is probably a major factor explaining these significant spectral changes. In Fig. 4, the fluorescence spectrum from $H4NO_2Fo4M$ in CH_2Cl_2 solution is shown.

Complexes (**1**)–(**3**) could not be dissolved in CH_2Cl_2, therefore, it was not possible to obtain fluorescence spectra from them in such medium. The use of a more polar solvent (DMF) enables the dissolution of these complexes; however, no fluorescence was observed from these solutions, possibly indicating either a dynamic quenching caused by DMF or an effective stabilization of the excited state bringing it to close to the fundamental state and therefore favoring radiationless energy deactivation.

These studies indicated that natural fluorescence from the thiosemicarbazones and their Mn(II) complexes may be used to identify and monitor these substances in biological systems.

The thiosemicarbazones and their complexes (**1**)–(**3**) have been deposited on a cellulose substrate previously treated with solutions of salts of Tl(I), Hg(II), Pb(II), Cd(II) or Ag(I) which are traditionally used as phosphorescence inducers from organic compounds. However, no phosphorescence was observed under these conditions.

3.6. In vitro anti-trypanosomal activity

Results showed that $H4NO_2Fo4M$, $H4NO_2Bz4M$, and complexes (**1**) and (**2**) were not active against bloodstream trypomastigotes, showing IC_{50} values higher that 142 μM (Fig. 5a, Table 3), while $H4NO_2Ac4M$ was moderately active (IC_{50} = 68 μM). However, complex (**3**) presented interesting trypanocidal activity, with IC_{50} = 19 μM and reaching 95% of parasite death at 50 μM (Fig. 5a, Table 3). It is important to notice, that the later complex showed quite similar *in vitro* trypanocidal effect upon bloodstream forms as compared to benznidazole, the reference drug for Chagas disease (Table 3). The effect of Mn(II) chloride salt was evaluated and the determined IC_{50} value of 340 μM indicates that Mn(II) does not possess anti-*T. cruzi* activity *per se* (Table 3).

The toxicity of the studied compounds towards mammalian host cells was also evaluated by employing primary cultures of cardiac cells. The results demonstrated that none of the thiosemicarbazone reduced the cellular viability even when higher doses (200 μM) and longer periods of incubation (72 h) were used.

The effect of the compounds was also evaluated in *T. cruzi*-infected cardiac culture assays aiming to further analyze their activity against the intracellular forms of the parasite. However, the thiosemicarbazones and their Mn(II) complexes did not present trypanocidal effect against intracellular parasites, exhibiting IC_{50} values between 111 and >200 μM (Fig. 5b, Table 3).

Further analysis of the selective index (SI) demonstrated that complex (**3**) was the most selective compound, being 10 times more active against bloodstream trypomastigotes than against mammalian cells (Table 3).

The lower activity of complex (**3**) against amastigotes (IC_{50} = 111 μM), a highly intracellular proliferative stage found within mammalian cells, as compared to its activity against bloodstream trypomastigotes may be explained by reduced ability of the compound to get into the host cells and/or by the fact that this compound may act on different targets according to the parasite stage.

Different efficacy was also noticed when Pt(II) complexes of 3-(5-nitrofuryl)acroleine thiosemicarbazones were assayed by other authors against both epimastigote and trypomastigotes of *T. cruzi*

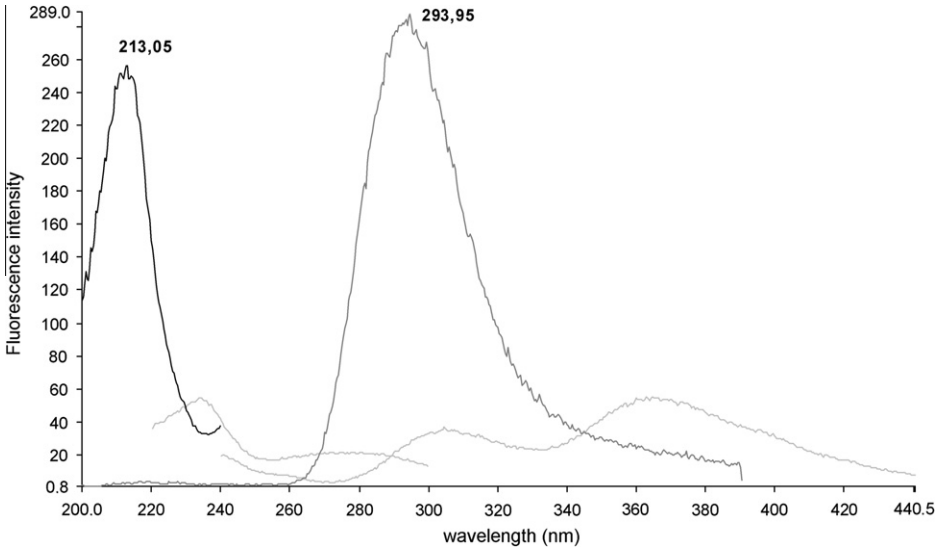

Fig. 4. Emission and excitation fluorescence spectra of $H4NO_2Fo4M$ in CH_2Cl_2 solution.

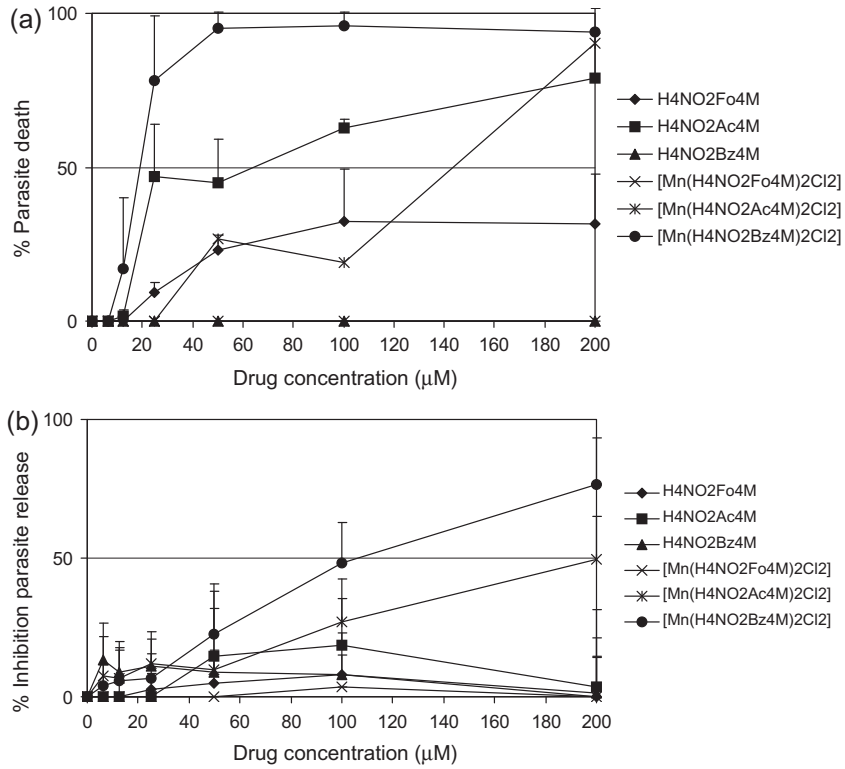

Fig. 5. Activity of $H4NO_2Fo4M$, $H4NO_2Ac4M$, $H4NO_2Bz4M$, $[Mn(H4NO_2Fo4M)_2Cl_2]$ (**1**), $[Mn(H4NO_2Ac4M)_2Cl_2]$ (**2**) and $[Mn(H4NO_2Bz4M)_2Cl_2]$ (**3**), upon bloodstream (a) and intracellular (b) forms of *T. cruzi in vitro*. *The effect upon parasites was evaluated during the treatment at 37 °C with the drugs diluted in culture medium. The percentage of dead bloodstream parasites was measured after 24 h of treatment (a) and the effect upon intracellular forms was evaluated by the quantification of parasite release in supernatant of untreated and drug-treated infected cultures after 72 h of treatment (b). Data are expressed as mean ± SD of three independent experiments.

Table 3
IC_{50} and respective SI* values for the thiosemicarbazones and their Mn(II) complexes upon bloodstream and intracellular forms of *Trypanosoma cruzi*.

Compound	BT[a]		Intracellular forms	
	IC_{50} (μM)	SI	IC_{50} (μM)	SI
$H4NO_2Fo4M$	>200	>1	>200	>1
$[Mn(H4NO_2Fo4M)_2Cl_2]$	>200	>1	>200	>1
$H4NO_2Ac4M$	68 ± 44	>3	>200	>1
$[Mn(H4NO_2Ac4M)_2Cl_2]$	142.5 ± 7	>1.4	200	>1
$H4NO_2Bz4M$	>200	>1	>200	>1
$[Mn(H4NO_2Bz4M)_2Cl_2]$	19.21 ± 0.1	>10	111 ± 29	>1.8
$MnCl_2{\cdot}4H_2O$	340 ± 83		nd	
Benznidazole [55]	10.92		nd	

* SI: selective index: ratio between LC_{50}/IC_{50} values. For bloodstream and intracellular forms the corresponding LC_{50} values were related to 24 and 72 h of drug incubation, respectively.
[a] Bloodstream trypomastigotes, nd: not done.

[41]. The activity of these complexes was superior against trypomastigotes as compared to epimastigotes [41]. As mentioned by these authors, it is important to consider the morphologic changes that occur during transformation between forms along the biological cycle of the parasite, which may imply important metabolic and macromolecular content changes that may alter sensibility to drugs [41].

In fact, the detailed mechanism of action of thiosemicarbazones is not fully understood and a bulk of data suggests that it may be mediated by multiple targets [19,21,42,43]. It has been shown that thiosemicarbazones inhibit ribonucleotide reductase, an enzyme essential for DNA synthesis [44–47]; also impairing dihydrofolate reductase [48,49]. There are also evidences that metal complexes of thiosemicarbazones may induce oxidative stress [22,50] and inhibit cysteine proteases [19]. Regarding *T. cruzi* although we presently did not explore the mechanisms of action of these nitro-thiosemicarbazones and their Mn(II) complexes, literature data showed that some active thiosemicarbazone derivatives hamper cruzain (aka cruzipain), which is the major cysteine protease of the parasite [43]. Cruzain is expressed in all evolutive stages of the parasite, playing a role in invasion, replication and differentiation steps [51–54]. Currently, cruzain inhibition is one of the most advanced and widely studied strategies in the design of new chemotherapeutic agents for the treatment of Chagas disease [51]. However, other *in vitro* studies also suggested that 5-nitrofuryl containing thiosemicarbazones that display higher trypanocidal activity than nifurtimox, could act both through the inhibition of cruzain and also by promoting oxidative stress in *T. cruzi* [50]. Thus, it is possible that these novel metal complexes of TS presently assayed may be also operating on these cellular targets of the parasite although others targets cannot be excluded justifying biochemical and electron microscopy studies that are underway to clarify this subject.

4. Conclusion

Despite the fact that many different classes of compounds have been assayed against *T. cruzi* both *in vitro* and *in vivo*, except for few of them, such as allopurinol, itraconazole and fluconazole, none moved to clinical trials [7]. Therefore, although clearly not of immediate clinical use, the non-peptide nature of the thiosemicarbazone group (i), its relative small size (ii), its extremely low cost of production (iii), its capacity to form the $Ar–NO_2^{\cdot-}$ radical in a potential range similar to that of nitro-containing anti-trypanosomal drugs (iv) and its trypanocidal efficacy (v) are positive arguments in favor that they represent promissory agents for the

development of much-needed new anti-trypanosomal drugs especially when coordinated to metal ions.

Complex (**3**) besides displaying higher efficacy than its corresponding thiosemicarbazone ligand upon bloodstream trypomastigotes, was 18-fold more effective than the Mn(II) salt. In addition, complex(**3**) showed the highest selective index, being as much as 10-fold more active against the trypanosomes than against the cardiac cells. Thus, the present study supports previous data reporting high trypanocidal effect induced by some metal complexes of thiosemicarbazones upon *T. cruzi* [18,24].

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