# ESR AND SPIN TRAPPING STUDIES OF TWO NEW POTENTIAL ANTITRYPANOSOMAL DRUGS

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## ABSTRACT

The Electron Spin Resonance (ESR) spectra of radicals obtained from two new potential antitrypanosomal drugs by *Trypanosoma cruzi* reduction were analyzed. DMPO Spin Trapping was used to investigate the possible formation of free radicals in the trypanosome microsomal system. The Nitro **2** (4-(n-butyl)-1-(5-nitrofurfurylidene)semicarbazide) analogue of Nifurtimox showed better antiparasitic activity than N-oxide **1** (4-(n-butyl)-1-[(7-bromo-N1-oxidebenzo[1,2-c]1,2,5-oxadiazole-5-yl)methylidene]semicarbazide). Only Nitro **2** could produce oxygen redox cycling in *T. cruzi* epimastigotes. The ESR signal intensities were consistent with the trapping of hydroxyl radical. These results are in agreement with the biological observation that Nitro **2** showed antichagasic activity by an oxidative stress mechanism.

**Keywords:** Nitrofuran derivatives, *N*-Oxide derivatives, ROS scavenging, ESR spin trapping, *T. cruzi*, oxidative stress

## 1. INTRODUCTION

Parasitic diseases in tropical and subtropical areas constitute a major health and economic problem. Chagas' disease, produced by several strains of *Trypanosoma cruzi,* affects approximately 24 million people from Southern California to Argentina and Chile [1]. Nifurtimox and benznidazole are currently used to treat this disease [2]. A characteristic ESR signal corresponding to the nitro anion radical ($R\text{-}NO_2\cdot$) appears when nifurtimox is added to intact *T. cruzi* cells [3]. This and other experiments [4-6] suggest that intracellular reduction of nifurtimox followed by redox cycling, yielding $O_2^{\cdot-}$ and $H_2O_2$, may be the major mode of action against *T. cruzi*. However, the use of nifurtimox has the disadvantage of its side effects [7].

Nitro compounds, especially 5-nitrofuryl derivatives, have been documented to be of great value as antiparasitic drugs. Recently we have explored 5-nitro-2-furaldehyde derivatives to find new substances with fewer side effects than Nifurtimox [8-12]. We have also carried out three-dimensional quantitative structure-activity relationship (3-D QSAR) studies on the *in vitro* and *in vivo* antiparasitic activities against *Trypanosoma cruzi* to establish the mode of action for this kind of semicarbazone derivatives [13,14].

In general, the biological effects of nitroheterocyclic compounds, especially in *T. cruzi*, involve redox cycling of these compounds and oxygen radical production, two processes in which the nitroanion radicals play an essential role [15].

Previously, we reported studies on the antiprotozoal activities of 5-nitrofurfural and 5-nitrothiophene-2-carboxaldehyde derivatives, and we showed that these compounds generate nitro anion radicals, characterized by ESR spectroscopy [16-17].

We also reported studies on the 1,2,5-oxadiazole *N*-oxide family in order to determine their antitrypanosomal activities, tested *in vitro* against the epimastigote form of *T. cruzi*. Moreover, we have shown some ESR spectra that prove the facile electronation of the *N*-oxide moiety. Beside, these new structures were based on the conjunction of *N*-oxide systems and the semicarbazide moieties ("spermidine-mimetic") [18]. In addition, we recently reported the electrochemical studies and the evidence of microsomal production of free radicals for 1,2,5-Oxadiazole *N*-Oxide suggesting its potential antiprotozoal activity [19]. All *N*-oxide studied showed similar $E_{1/2}$ to nifurtimox. Additionally, the side chains assessed in this work did not modify the $E_{1/2}$, an aspect that might be important for the selectivity of these compounds towards trypanothione reductase. Stable free radicals generated using a microsomal system showed hyperfine coupling constants identical to those of the radicals obtained by electrochemical reduction. The ESR spectra also proved that the *N*-oxide group is protonated, as suggested by the reduction mechanism proposed from the cyclic voltammetric results.

In the present study, we report the ESR and spin trapping results of two new potential antitrypanosomal drugs: 4-(n-butyl)-1-[(7-bromo-*N*$^1$-oxidebenzo[1,2-c]1,2,5-oxadiazole-5-yl)methylidene]semicarbazide (N-oxide**1**) and 4-(n-butyl)-1-(5-nitrofurfurylidene)semicarbazide (Nitro**2**) (Figure 1). Both molecules have the same side chain but with different groups generating free radical species such as the nitro and *N*-oxide groups. In this paper we have characterized the free radical species generated by *T. cruzi* reduction that correlate with the percentage of growth inhibition of *T. cruzi* epimastigotes in order to suggest a possible mechanism.

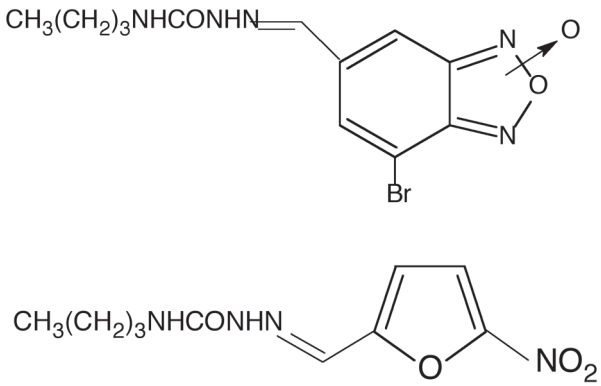


**Fig. 1.-** Chemical structure of the potential antitrypanosomal drugs.

## 2. EXPERIMENTAL SECTION AND THEORETICAL METHODS

**2.1.** ***Samples.***

The N-oxide **1** and Nitro **2** were synthesized according to methods described earlier [9, 20].

**2.2.** ***Reagents***

Dimethylsulfoxide (DMSO) (spectroscopy grade), glutathione (GSH), 5,5-dimethyl-1-pyrroline N-oxide (DMPO), reduced Nicotinamide Adenine Dinucleotide Phosphate (NADPH), ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma Aldrich Co., St. Louis, MO. Tetrabutylammonium perchlorate (TBAP) used as supporting electrolyte was obtained from Fluka.

**2.3.** ***ESR Spectroscopy*****.**

ESR spectra were recorded in the X band (9.85 GHz) using a Bruker ECS 106 spectrometer with a rectangular cavity and 50 kHz field modulation. The hyperfine splitting constants were estimated to be accurate within 0.05 G. ESR spectra of the anion radical drugs and the radical oxygen species were produced using a microsomal fraction (4 mg protein/mL) obtained from *T. cruzi*, in a reaction medium containing 1mM NADPH, 1mM EDTA and 100 mM DMPO, in 20mM phosphate buffer, pH 7.4. The ESR spectra were simulated using the program WINEPR Simphonia 1.25 version.

**2.5.** ***Parasites***

*Trypanosoma cruzi* epimastigotes (Tulahuen strain), from our collection, were grown at 28 ºC in Diamond's monophasic medium as reported earlier [21,22], with blood replaced by 4 mM hemin. Fetal calf serum was added to a final concentration of 4%. Parasites: 8 x $10^7$ cells correspond to 1 mg protein or 12 mg of fresh weight.

## 3.0 RESULTS AND DISCUSSION

**3.1** ***ESR***

We have obtained a well resolved ESR spectra for both anion radical derivatives when they are prepared *in situ* by electrochemical reductions in DMSO, applying a potential corresponding to the first wave as obtained from the cyclic voltammetric experiments (data not shown). However, when *T. cruzi* microsomes are incubated with both compounds, after a 10 min induction period to become the microsomes anaerobic, gave a not well-resolved ESR spectra (data not shown), probably attributable to both radical species.

**3.2** ***Effect of Nitro 2 and N-Oxide 1 upon culture growth in Trypanosoma cruzi epimastigotes***

Several drug concentrations were used in order to determine the respective $IC_{50}$ Nitro **2** produced significant inhibition of epimastigote culture growth ($IC_{50}$ of 7.4±0.5 µM). N-oxide **1** showed a much lower effect upon epimastigote growth ($IC_{50}$ > 30 µM).

**3.3** ***ESR spectra of DMPO-OH· and DMPO-N-Oxide adducts obtained with T. cruzi extracts***

In order to analyse the antitrypanosomal mechanism of these drugs, we incubated both compounds with *T. cruzi* homogenates in the presence of NADPH and EDTA and DMPO (figures 2 and 3). A well-resolved ESR spectrum appeared when DMPO was added to the *T. cruzi*-Nitro **2** system. The ESR signal intensity was consistent with the trapping of the hydroxyl radical (DMPO-OH spin adduct $a_N$=$a_H$ = 14.7 G) (Figure 2). These hyperfine constants are in agreement with the splitting constants of other DMPO-OH adducts by DMPO [23]. These results are in agreement with the above-mentioned activity for this compound. However, when the N-oxide **1** compound was incubated with *T. cruzi* in presence of DMPO (figure 3), six ESR line appeared. According with this hyperfine pattern and the hyperfine constants ($a_N$ = 15.6 G and $a_H$ = 21.6 G ) this spectrum was consistent with the trapping of the *N*-oxide radical (Figure 3), which can not produce radical oxygen species. These results agree with the low activity of these compounds. On the other hand, *N*-oxide-produced low growth inhibition of *T. cruzi* occurs at concentrations that do not stimulate hidroxyl radical generation. As seen in their structures, both molecules have the same side chain, which could indicate that the nitro group generated radical species more efficiently than *N*-oxide group.

Finally, for Nitro **2** the main toxic mechanism seems to be the production of oxidative stress because of the extensive redox cycling that Nitro **2** undergoes.

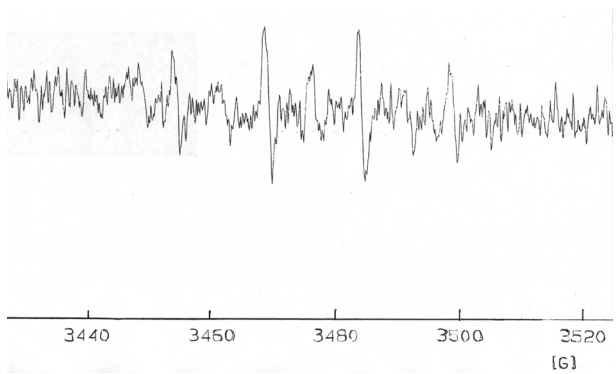


**Fig. 2.-** ESR spectra of DMPO-OH· adduct obtained with *T. cruzi* extracts with Nitro **2**. The ESR spectra were observed 10 min after incubation at 37°C with *T. cruzi* microsomal fraction (4 mg protein/mL), NADPH (1mM), EDTA (1mM), in phophate buffer (20mM), pH 7,4 , DMPO (100mM), Nitro 2 (1mM in acetonitrile 10 v/v). Spectrometer conditions: microwave frequency 9.68 GHz microwave power 20 mW, modulation amplitude 0.4G, scan rate 0.83 G/s , time constant 0.25 s number scans: 10.

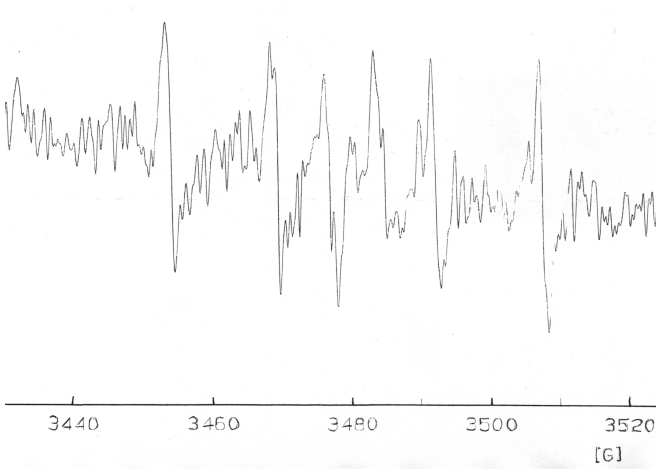


**Fig. 3.-** ESR spectra of DMPO-N-oxide adduct obtained with *T. cruzi* extracts with N-oxide **1**. The ESR spectra were observed 10 min after incubation at 37°C with *T. cruzi* microsomal fraction (4 mg protein/ mL), NADPH (1mM), EDTA (1mM), in phophate buffer (20mM), pH 7,4 , DMPO (100mM), N-oxide **1** (1mM in acetonitrile 10 v/v). Spectrometer conditions: microwave frequency 9.68 GHz microwave power 20 mW, modulation amplitude 0.4G, scan rate 0.83 G/s , time constant 0.25 s number scans: 10.

## CONCLUDING REMARKS

The ESR spectra of the anion radicals for N-oxide **1** and Nitro **2** generated by *T. cruzi* system showed low resolution. However, well resolved ESR spectra were obtained when DMPO was added to the system. For Nitro **2**, the ESR signal intensity was consistent with the trapping of the hydroxyl radical, and for the N-oxide **1,** its spectrum was consistent with the trapping of the *N*-oxide radical.

The biological studies and the ESR experiment with the *T. cruzi* system indicate that Nitro **2** and N-oxide **1** could have different mechanisms of toxicity. While Nitro **2** may act by production of oxidative stress throughout the increase in redox recycling of the molecule; N-oxide **1** seems to act through different inhibition mechanism.

## ACKNOWLEDGEMENTS

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