ESR, electrochemical and reactivity studies of antitrypanosomal palladium thiosemicarbazone complexes

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Abstract

Cyclic voltammetry (CV) and electron spin resonance (ESR) techniques were used in the investigation of novel palladium complexes with bioactive thiosemicarbazones derived from 5-nitrofurane or 5-nitrofurylacroleine. Sixteen palladium complexes grouped in two series of the formula $[PdCl_2HL]$ or $[PdL_2]$ were studied. ESR spectra of the free radicals obtained by electrolytic reduction were characterized and analyzed. The ESR spectra showed two different hyperfine patterns. The stoichiometry of the complexes does not seem to affect significantly the hyperfine constants however we observed great differences between 5-nitrofurane and 5-nitrofurylacroleine derivatives. The scavenger properties of this family of compounds were lower than Trolox.

Keywords: ESR; Chagas; Pd complex; ORAC; Cyclic voltammetry

1. Introduction

Infections caused by trypanosomatid protozoa are among the most important parasitic diseases in the world and are responsible for heavy socioeconomic losses, especially in underdeveloped countries. In particular, over 18 million people are infected and over 90 million are at risk of infection by *Trypanosoma cruzi* (*T. cruzi*), causative agent of Chagas' disease [1,2]. Despite the progress achieved in the study of *T. cruzi* biochemically and physiologically in recent decades, the drugs that are commercially available for the treatment of this disease have been those discovered empirically 40 years ago: Nifurtimox and Benznidazole. These drugs have significant activity only in the acute phase of the disease. Long-term treatments are known to rise to severe side effects [3,4]. Therefore, the urgent need for more efficacious and safe chemotherapeutic approaches for the treatment of Chagas' disease is evident.

In this sense, metal complexes appear to be a promising alternative in the search of a pharmacological answer to Chagas' disease. The several activities of metal ions respoted in biology have stimulated the development of metal-based chemotherapeutics in different fields of medicine. Even though emphasis has been placed mainly on the cancer treatment as a result of the great success of cisplatin. Recent studies have also included parasitic diseases [5–8]. One of the most successful developed approaches has been the synthesis of complexes combining ligands showing antitrypanosomal activity and bearing pharmacologically active metals. Thereby, the obtained compounds could act through a dual mechanism of action combining the pharmacological properties of the ligands and the metals [7].

Based on this approach, our group has developed a series ligands with of rhenium, ruthenium and palladium complexes

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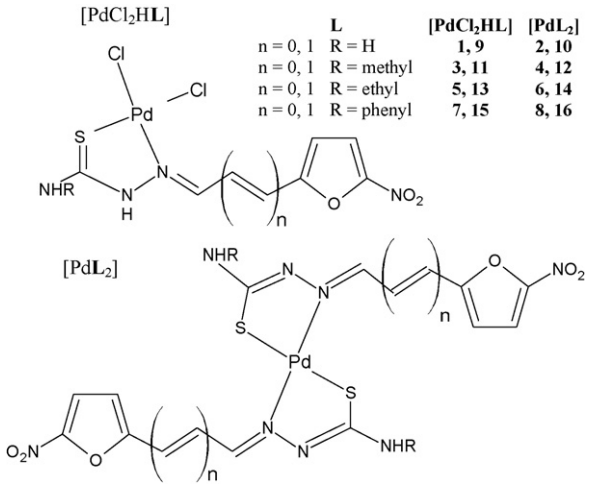

Fig. 1. Palladium complexes of the selected 5-nitrofuryl containing thiosemicarbazones.

showing anti-*T. cruzi* activity that contain the ligand 5-nitrofuryl pharmacophore [9–12]. These main modes of action, of these like Nifurtimox's or other nitroheterocycle antiparasitic agents, are related to the intracellular reduction of the nitro moiety followed by redox cycling, yielding reduced oxygen species (ROS). These can cause cellular damage directly by reacting with various biological macromolecules, or indirectly by generation of the highly reactive hydroxyl radical via iron-mediated Haber-Weiss and Fenton reactions. Due to the lack of catalase and glutathione peroxidase, trypanosomes have an impaired enzymatic defense against ROS, thus they have long been deemed specially sensitive to oxidative stress [13–15].

In particular, we have described novel palladium complexes with bioactive thiosemicarbazones derived from 5-nitrofurane or 5-nitrofurylacroleine. Sixteen palladium complexes grouped in two series of the formula $[PdCl_2HL]$ and $[PdL_2]$ (Fig. 1), where HL is the neutral thiosemicarbazone ligand and L the monodeprotonated one, were synthesized and characterized. The obtained complexes were, in most cases, more active *in vitro* against *T. cruzi* than Nifurtimox. The biological activity of each ligand was maintained or increased as a result of palladium complexation. Even though all obtained complexes bind DNA, their main toxic effect on the parasite seems to be related to redox metabolism. All performed experiments strongly suggest that the main mechanism underlying the trypanocidal activity of the complexes is the production of oxidative stress as a result of their bioreduction and extensive redox cycling [12].

In this work, we report the electrochemical and electron spin resonance (ESR) studies of the sixteen novel palladium(II) complexes shown in Fig. 1. In order to study the radical species involved in the mechanism of parasitic toxicity, the formal one-electron-transfer potential for the new palladium complexes was compared with that of Nifurtimox and the nitroanion radical, produced in the electrochemical process, was characterized by ESR. The study of the free-radical scavenger properties for this family, oxygen radical absorbance capacity (using fluorescein (FL) as probe, $ORAC_{FL}$) studies were performed.

2. Experimental

2.1. Samples

The palladium(II) complexes were synthesized according to previously described methods [12].

2.2. Reagents

Dimethylsulfoxide (DMSO) (spectroscopy grade), tetrabutylammonium perchlorate (TBAP) used as supporting electrolyte, fluorescein disodium salt (FL), 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), were obtained from Fluka.

2.3. Cyclic voltammetry

Cyclic voltammetry (CV) was carried out using a Metrohm 693 VA instrument with a 694 VA Stand convertor and a 693 VA Processor in DMSO (ca. 1.0×10^{-3} M) under a nitrogen atmosphere at room temperature with TBAP (ca. 0.1 M), using a three-electrode cell. A hanging drop mercury electrode (HDME) was used as the working electrode, a platinum wire as the auxiliary electrode, and saturated calomel (SCE) as the reference electrode.

2.4. Electron spin resonance 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. The palladium complexes' radicals were generated by electrolytic reduction *in situ* at room temperature. ESR spectra of the anion radicals were obtained from the electrolysis solution. The ESR spectra were simulated using the program WINEPR Simphonia 1.25 version.

2.5. $ORAC_{FL}$ assay

A luminescence spectrometer LS 50B (Perkin-Elmer, Boston, MA, USA), a heating circulator bath DC1–B3 (Haake Fisons, Karlsruhe, Germany) and quartz cuvettes were used. For the $ORAC_{FL}$ assay, the 490-P excitation and 515-P emission filters were used, and the fluorescence measurement was carried out at 60 °C. *ORAC procedure*: The method of Ou et al. [16] was modified as follows. The reaction was carried out in 75 mM phosphate buffer (pH 7.4), and the final reaction volume was 3000 μL. Studied compounds (15, 30, 45, 60 μL; 0.5–2.0 μM final concentrations) and FL (215 μL; 70 nM final concentration) solutions were placed in the quartz cuvette. The mixture was preincubated for 30 s at 60 °C. AAPH solution (240 μL; 12 mM, final concentration) was added rapidly using a single channel pipette. The quartz cuvette was immediately placed in the luminescence spectrometer and the fluorescence recorded every minute for 12 min. As a blank FL plus AAPH in phosphate buffer instead of the studied compounds solutions were employed and eight calibration solutions using Trolox (1–8 μM,

final concentration) as antioxidant positive control were also carried out in each assay. All the reaction mixtures were prepared in duplicate, and at least three independent assays were performed for each sample. Raw data were exported to an OriginPro (OriginLab Corporation, Northampton, MA) sheet for further calculations. Blank and antioxidant curves (fluorescence versus time) were first normalized by dividing original data by fluorescence at $t = 0$ s. From the normalized curves, the area under the fluorescence decay curve (AUC) was calculated as $\mathrm{AUC} = 1 + \sum_{i=1}^{i=12} f_i/f_0$ where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time i. The net AUC corresponding to each sample was calculated by subtracting the AUC corresponding to the blank. Regression equations between net AUC and antioxidant concentration were calculated for all the samples. $\mathrm{ORAC_{FL}}$ values were expressed as Trolox equivalents by using the standard curve calculated for each assay. Final results were expressed in μmol of Trolox equivalent/μmol of samples [17].

3. Results and discussion

3.1. Cyclic voltammetry

Fig. 2a and b shows the voltammograms displayed for compounds **11** and **12** of both series of palladium complexes in DMSO solution (1 mM, TBAP 100 mM) swept from 0 to −2.0 V. We noticed clearly a one-electron reversible transfer (peak IIc/IIa, Fig. 1) corresponding to the generation of the radical anion $\mathrm{RNO_2^{\bullet -}}$ around −0.80 V. The stability of these radical intermediates changed with the electrochemical conditions, *i.e.* the scan rate, while keeping the chemical conditions of the solution unaltered. We observed that the ipa/ipc ratio calculated with the Nicholson and Shain equation [18] increases slightly as the scan rate increases (from 100 to 2000 mV/s) as is typical for a reversible charge transfer (results not shown) [19]. For some complexes, sharp peaks around this couple could be observed as a result of adsorption phenomena in the electrode surface due to the presence in the molecules of the thiocarbonyl group and Pd metal (results not shown).

Table 1 lists the values of voltammetric peaks for all compounds under study. All complexes exhibited lower $E_{1/2}$ ($E_{1/2} = (E_a + E_c)/2$) values than Nifurtimox (−0.90 V [20]). They show a higher capacity to be reduced and a better ability to generate the radical species. Similar potential values were found for the free ligands [21]. All complexes with one coordinated nitroheterocyclic ligand ($[PdCl_2HL]$), **1**, **3**, **5**, **7**, **9**, **11**, **13**, and **15**, displayed comparable voltammetric behaviour in DMSO however small differences were found when the complexes have two coordinated nitroheterocyclic ligands ($[PdL_2]$), **2**, **4**, **6**, **8**, **10**, **12**, **14**, and **16**. In general, Pd complexes showed subsequent less negative three-electron irreversible cathodic peak (IIIc, Fig. 2a and b) belonging to the production of the hydroxylamine derivative [22] that resulted irreversible in the whole range of sweep rates used (100–2000 mV/s). The voltammogram of $[PdCl_2HL]$ complexes showed a prepeak (Ic, Fig. 2a) that appears even before the reduction of the nitro group, meaning that the nitro group follows another reaction path besides the

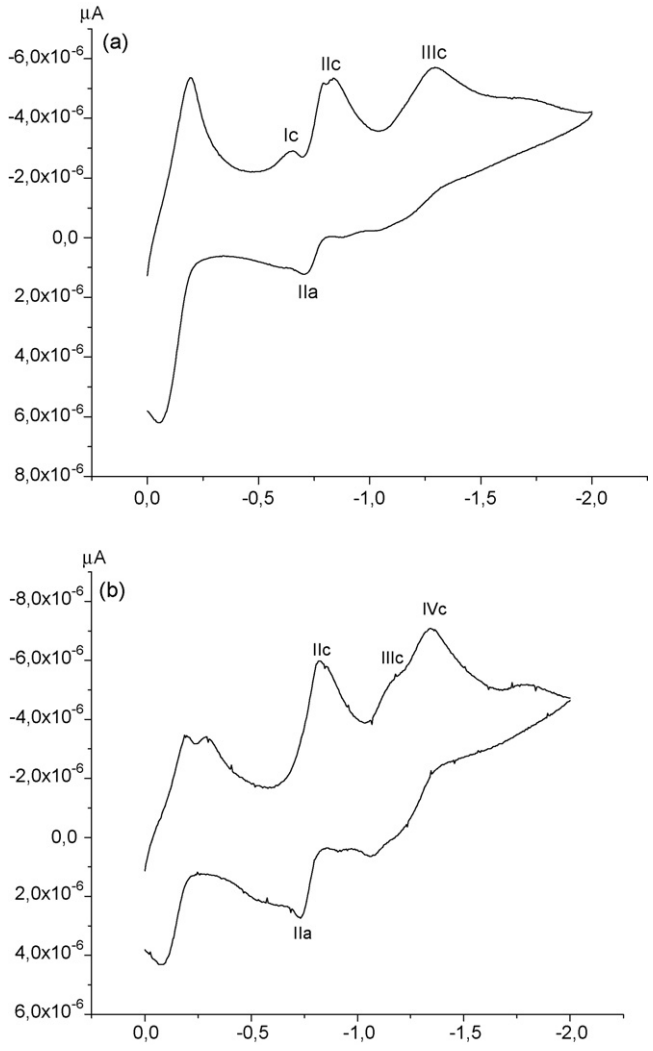

Fig. 2. (a) Cyclic voltammetry of complex **11** in DMSO at 2000 mV/s. (b) Cyclic voltammetry of complex **12** in DMSO at 2000 mV/s.

Table 1
Cyclic voltammetric parameters for the reduction of the palladium complexes corresponding to the couple II, peak Ic, IIIc and IVc measured in DMSO at 2000 mV/s

Compound	E_{pIc}	E_{pIIc}	E_{pIIa}	E_{pIIIc}	E_{pIVc}
1	−0.62	−0.83	−0.70	−1.29	−1.54
2	–	−0.82	−0.72	−1.30	−1.57
3	0.62	−0.82	−0.70	1.27	−1.41
4	–	−0.84	−0.75	−1.30	–
5	−0.60	−0.71	−0.70	−1.40	–
6	–	−0.79	−0.69	−1.40	–
7	−0.59	−0.74	−0.69	−1.14	−1.41
8	–	−0.73	−0.69	−1.13	−1.30
9	−0.59	−0.78	−0.68	−1.17	–
10	–	−0.77	−0.71	−1.28	–
11	−0.65	−0.81	−0.71	−1.29	–
12	–	−0.80	−0.73	−1.33	–
13	−0.66	−0.84	−0.73	−1.20	−1.35
14	–	−0.89	−0.64	−1.38	–
15	−0.63	−0.81	−0.76	−1.22	–
16	–	−0.81	−0.75	−1.22	–

Potentials are reported in volts *vs.* saturated calomel electrode.

known electron-transfer mechanism of the nitroaromatic compounds in aprotic media. This prepeak would correspond to the four-electron reduction of a small portion of the molecules reaching the electrode surface, while the remaining portion would supply the protons required for this reduction. The presence of the nitro group increases the acidity of the NH moiety of the thiosemicarbazone group which becomes capable of protonating the nitro group of a minor part of the molecules in the solution, resulting into a lower intensity of these signals. This is a typical behaviour of a self-protonation phenomenon displayed by nitrocompounds with acidic moieties in their structures [20–22]. For $[PdL_2]$ complexes the Ic prepeak was not observed because these complexes do not have the capability to protonate the nitro group due to that NH proton was lost as a consequence of palladium coordination in these complexes (Fig. 1b). Peak IVc observed in some complexes is presumed to belong to the reduction of the imine moiety (CH=N) of the thiosemicarbazone group [23]. Peaks not labeled in Fig. 2 correspond to the reduction of the supporting electrolyte (TBAP).

3.2. ESR

The electrochemical reductions to the radical forms (*in situ*) in DMSO were carried out applying the potential corresponding to the IIc/IIa wave for the palladium complexes, obtained from the CV experiments. The interpretation of the ESR spectra by means of a simulation process has led to the determination of the coupling constants for all magnetic nuclei. The obtained hyperfine constants are listed in Table 2.

Two different hyperfine patterns were found for this family of compounds. The number of ligands does not seem to affect significantly the hyperfine constants, however we observed great differences between complexes with 5-nitrofurane ($n=0$, compounds **1**–**8**, Fig. 1) and 5-nitrofurylacroleine ($n=1$, compounds **9**–**16**, Fig. 1) derivatives as ligands. The ESR spectrum of palladium complexes with ligands having the shortest chain ($n=0$) were simulated in terms (i) two triplets corresponding to the nitrogen nucleus belonging to the nitro group and the C=N thiosemicarbazone group, (ii) three doublets due to non-equivalent hydrogens belongs side chain; and (iii) one doublet due to the hydrogen which hyperfine constant are smaller than the line width. Fig. 3a shows the ESR spectrum of complex **1**. The ESR spectra of palladium complexes with ligands having the longest chain ($n=1$) were analyzed in terms of (i) one triplet due to the nitrogen of the nitro group; (ii) two doublets due to the hydrogens corresponding to the furan ring; and (iii) signals corresponding to hydrogens and one nitrogen, C=N, belonging to the thiosemicarbazone chain whose hyperfine constants resulted smaller than the line width and therefore they were not observed in the experimental spectrum. Fig. 3b shows the ESR spectrum of complex **9**.

These two hyperfine different patterns found for these complexes could be explained according to the different structures of the furane side chain [24]. In compounds **9**–**16** the flexibility and therefore the different conformational situations of the olefinic system, –CH=CH–CH=N–, could decrease the optimal orbital overlapping in the lateral chain, diminishing the coupling of

Table 2
Hyperfine constants (Gauss) for the anion radical of palladium complexes

Compound	aN		aH			
1	9.5	4.5	1.2	1.5	5.0	0.8
2	9.2	4.7	1.2	1.6	4.8	0.6
3	9.5	4.6	1.1	1.5	5.0	0.7
4	9.2	5.0	1.1	1.8	4.6	0.6
5	8.8	4.5	1.0	1.6	4.5	0.7
6	9.5	4.4	1.2	1.5	5.0	0.7
7	8.0	4.0	1.2	1.0	4.5	<0.6
8	11.0	4.5	1.1	1.0	5.5	<0.6
9	8.5	<0.6	1.2	4.5	<0.6	<0.6
10	9.0	<0.6	1.5	5.0	<0.6	<0.6
11	8.8	<0.6	1.2	4.8	<0.6	<0.6
12	8.6	<0.6	1.1	5.0	<0.6	<0.6
13	9.0	<0.6	1.5	5.0	<0.6	<0.6
14	8.5	<0.6	1.2	4.5	<0.6	<0.6
15	9.2	<0.6	1.2	5.1	<0.6	<0.6
16	8.8	<0.6	1.2	4.5	<0.6	<0.6

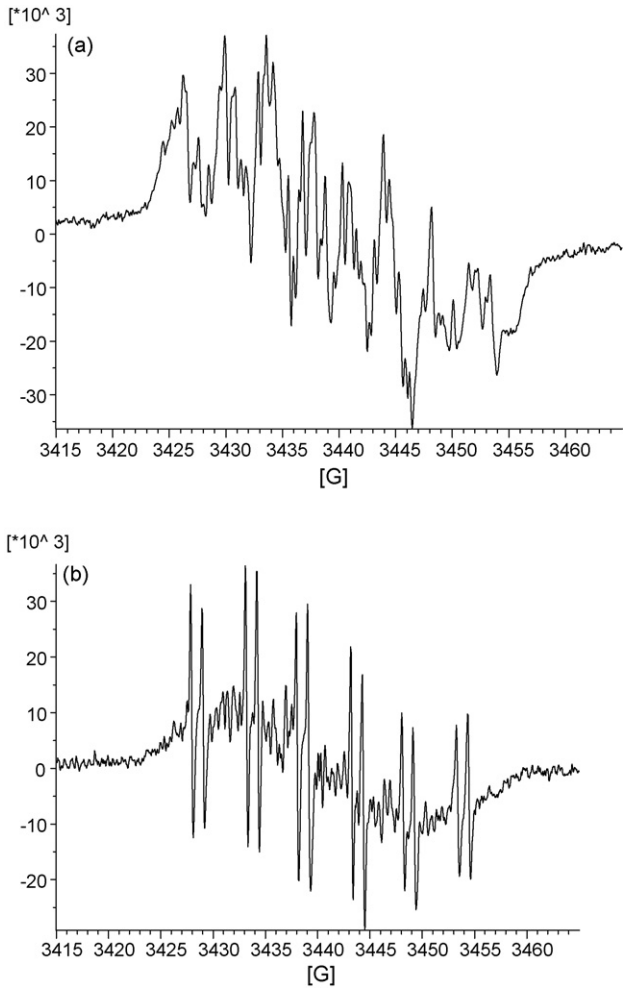

Fig. 3. (a) ESR experimental spectrum of complex **1** radical produced by an electrochemical generation in DMSO with a 1 mM solution of the complex in 100 mM TBAP. (b) ESR experimental spectrum of complex **9** radical produced by an electrochemical generation in DMSO with a 1 mM solution of complex in 100 mM TBAP. *Spectrometer conditions*: Microwave frequency, 9.71 GHz; microwave power, 20 mW; modulation amplitude, 0.2 G, scan rate, 1.25 G/s; time constant, 0.5 s; number of scans 15.

Table 3
Trolox equivalents of the studied palladium complexes

Compound	Trolox-equivs[a,b]	r^2
1	1.49 ± 0.20	0.980
2	1.78 ± 0.05	0.984
3	1.43 ± 0.09	0.988
4	1,96 ± 0.14	0.958
5	1.86 ± 0.09	0.990
6	1.60 ± 0.12	0.980
7	–[c]	–
8	–	–
9	1.58 ± 0.1	0.972
10	–	–
11	1.35 ± 0.06	0.998
12	1.06 ± 0.08	0.998
13	1.69 ± 0.2	0.961
14	1.44 ± 0.1	0.930
15	–	–
16	–	–
Trolox	1.00 ± 0.02	0.997

[a] Expressed as μmol of Trolox equivalent/μmol of compounds.
[b] Results are presented as the mean ($n = 3$) ± S.D.
[c] Solubility problems.

these atoms with the unpaired electron (see hyperfine constants in Table 2). The spin density is more localised in the nitrofurane ring. Palladium coordination through the iminic nitrogen and the thiocarbonyl group would not affect the long side chain flexibility. In compounds **1**–**8** the conjugation are located in the iminic system, –CH=N–. The theoretical study for the ligand indicated that the short side chain adopted folded conformation stabilised by the presence of hydrogen bond, while the long chain adopted an extended conformation. The complexes have the same conformation of free ligands [24].

3.3. ORAC assay

In order to study the effect of the hydrogen donating ability of the complexes and its relationship with the trypanosomicidal activity, the free-radical scavenger capacity of palladium complexes **1**–**16** was further evaluated using the ORAC assay with $ROO^{\bullet}$ species. The regression and correlation coefficients are given in Table 3. The results indicated that the scavenger properties of this family of compounds were lower than for Trolox. Complex **12** showed the highest relative antioxidant activity toward $ROO^{\bullet}$. Thus, the trypanocidal activity of this series of compounds could not be correlated with the free-radical scavenger properties.

4. Concluding remarks

All palladium complexes studied showed lower E_{pc} IIc/IIa potentials than the Nifurtimox which could favour their antitrypanosomal activity. A self-protonation process involving the protonation of the nitro group due to the presence of an acidic proton in the thiosemicarbazone moiety was observed in $[PdCl_2HL]$ complexes.

The stoichiometry of the complexes does not seem to affect significantly the hyperfine constants. However, we observed great differences when the length of the side chain is changed. These differences could be explained by the different geometry that these side chains could adopt in the complexes and the hyperfine constants would also be affected by the coordination to palladium. Besides, the results indicated that the scavenger properties for this family of compounds were low and that they could not be correlated with the trypanocidal activity of this series. Finally the palladium complexes showed a different conformation and spectroscopic behaviour which is dependent of the stoichiometry and structure. This family could be a well target to joust Chaga's disease.

Acknowledgments

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