Original article

New oxidovanadium(IV) N-acylhydrazone complexes: Promising antileishmanial and antitrypanosomal agents

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Abstract

Searching for new promising metal-based hits against Trypanosoma cruzi and Leishmania parasites, two related oxidovanadium(IV) N-acylhydrazone complexes, [VIVO(LASSBio1064-2H)(H2O)], 1, and [VIVO(LASSBio1064-2H)(phen)](H2O), 2, where LASSBio1064 = (E)-N-(2-hydroxybenzylidene-4-chlorobenzohydrazide and phen = 1,10-phenanthroline, were synthesized and characterized in the solid state and in solution by elemental analysis, conductimetric measurements and ESI-MS, FTIR, EPR and 51V NMR spectroscopies and were evaluated on T. cruzi and Leishmania major. In addition, their unspecific cytotoxicity was tested against murine macrophages. Furthermore, to provide insight into the possible mechanism of its antiparasitic action, [VO(LASSBio1064-2H)(phen)](H2O) was tested for its DNA interaction ability on plasmid DNA by atomic force microscopy (AFM) and on CT DNA by using DNA viscosity measurements and fluorescence spectroscopy. Both complexes were active in vitro against the epimastigote form of T. cruzi (Tulahuen 2 strain) showing IC50 values of the same order or significantly lower than that of the reference trypanosomicidal drug Nifurtimox. However, only the mixed-ligand oxidovanadium(IV) complex 2, which includes phen in its coordination sphere, showed activity on L. major promastigotes with an IC50 value of 22.1 ± 0.6 M. The compounds show low toxicity on mammalian cells (IC50 > 100 M). DNA interaction studies showed that the mixed-ligand complex is able to interact with this biomolecule probably through an intercalative mode, pointing out at DNA as a potential target in the parasite. The results suggest that [VIVO(LASSBio1064-2H)(phen)](H2O) may be a promising compound for further drug development stages.

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1. Introduction

Chagas disease (American Trypanosomiasis) and Leishmaniasis are considered by the World Health Organization as neglected diseases (NTDs) and constitute important health problems concentrated in the poorest regions of the planet [1–3]. Both are among the NTDs with the highest statistics of death [1]. They are produced by genetically related protozoan parasites that belong to the trypanosomatid genus and kinetoplastid order, Trypanosoma cruzi and Leishmania spp., respectively, and are mainly transmitted to the mammalian host by the bite of certain insects [4–6].

The chemotherapy of both diseases is far from being adequate and mostly relies on drugs that date back to over 40–50 years having poor efficacy and/or high toxicity, and increasing resistance development [2–4,7]. New drugs against both diseases are thus urgently needed and efforts are being made by medicinal chemists to identify new organic or metal-based compounds that may be leads or hits for further antiparasitological or antileishmanial drug development [8,9].

The N-acylhydrazones (RCONHN=CHR), NAH, are simple molecular frameworks synthetically obtained from functional group...
interconversions, exploiting carboxylic acid derivatives as starting materials, that have been reported as ‘privileged structures’ [10,11]. In this context several pharmacological activities were reported for NAH derivatives including antimicrobial, anticonvulsant, analgesic, anti-inflammatory, antiplatelet, antiviral, antibacterial, antitumoral and antiparasitic activities, among others [12]. Pursuing treatment of parasitic diseases changes in the nature of the substituents attached to the acyl and imine groups of N-acylhydrazone subunit has allowed the identification of lead-compounds for the treatment of leishmaniasis, sleeping sickness and Chagas disease [13–15].

The development of bioactive metal complexes is currently also considered a promising approach in the search for new potential drugs for the therapy of parasitic illnesses, particularly malaria, leishmaniasis and trypanosomiasis [6,7,16–21]. In this path, some bioinorganic chemistry groups are orienting their research toward the development of trypanosomicidal metal-based compounds [6,7,18–27]. In particular, some of us have been successfully working on potential antitypansomonal and antileishmanial agents through different approaches using different metal centers [Pd(II), Pt(II), Ru(II,III), V(IV,V), Au(I), and different 3d M(II) ions] [19–21,28–33]. One of the strategies is based on the knowledge that highly-proliferative cells such as kinetoplastid parasites efficiently interact with DNA in an intercalative mode also show antileishmanial and/or antitypansomonal activity [6,20,34]. The aim of this strategy is to develop antitypansomonal metal compounds by including DNA intercalators as ligands in the metal ion coordination sphere, pointing out to DNA as potential target [19–21].

Although the potentiality of vanadium compounds in medicinal chemistry and medicinal applications has been extensively explored, research has been mainly focused whether on improving biodistribution and tolerability of the vanadium insulin-enhancing core or on developing potential anti-tumor compounds [35,36]. Work on vanadium compounds for the treatment of some parasitic diseases of high incidence in human health has only arisen in a systematic way in recent years [20]. In particular, our research in this area led to really encouraging results for the vanadium complexes when compared to other metal compounds [20,28,29].

Having this background in mind, some of us have focused their research on the development of potential vanadium-based antitypansomomatid drugs. Several homoleptic and heteroleptic oxidovanadium(IV) complexes including DNA intercalators as ligands (dppz = dipipyrido[3,2-a: 2’,3’-c]phenazine, bipy = 2,2’-bipyridine, phen = 1,10-phenanthroline) have been developed. [VO(O(SO4)2)(H2O)]2(dpdz)]·2H2O and mixed-ligand oxidovanadium(IV) complexes, [VO(O(L2-2H)(L1)], including a bidentate pyridyl DNA intercalator (L1 = dpdz, bipy, phen) and a tridentate salicylddehyde semicarbazone derivative (L2) as ligands showed IC50 values in the micromolar range against Dm28c strain (epimastigotes) of T. cruzi, being slightly more active than the reference trypanocidal drug Nifurtimox. By using atomic force microscopy, gel electrophoresis, circular dichroism and fluorescence spectroscopic experiments and DNA viscosity measurements evidence was obtained pointing out at DNA as a potential target of these compounds [20,32,37,38].

Further work in this research area led to the development of two related oxidovanadium(IV) N-acylhydrazone complexes including a NAH designed by the LASSBio group, code LASSBio-1064, as ligand (Fig. 1). A binuclear Zn(II) LASSBio-1064 complex, [Zn2(LASSBio-1064)2] was previously reported [37], and in the present work two new complexes [VO(O(LASSBio1064-2H)(H2O))] and [VO(O(LASSBio1064-2H)(phen)]·(H2O) were prepared and characterized in the solid state and in solution and tested in vitro on T. cruzi epimastigotes and Leishmania major promastigotes. In addition, their unspecific cytotoxicity was tested against murine macrophages. Furthermore, to provide insight into the possible mechanism of antiparasitic action, [VO(O(LASSBio1064-2H)(phen)]·(H2O) was tested for its DNA interaction ability by using different techniques.

2. Results and discussion

Two novel oxidovanadium(IV) complexes including the NAH ligand LASSBio-1064, [VO(O(LASSBio1064-2H)(H2O))] and [VO(O(LASSBio1064-2H)(phen)]·(H2O), 2, were synthesized with high purity and reasonable yields (Fig. 1). Although both include the same NAH ligand, one of them also includes 1,10-phenanthroline as co-ligand. Both of them are neutral non conducting compounds in DMF. Analytical, ESI mass spectrometry and FTIR and EPR
spectroscopic results are in agreement with the proposed structures. The ESI-MS spectra confirm the formulas proposed for the synthesized complexes. These experiments allowed the clear detection of the protonated complex ion, $M + H^+$, for compound 2.

For the solution of complex 1 we assign the peak observed at $m/z = 379.3$ (100%) to $[\text{V}^{IV}(\text{LASSBio-1064-2H})\text{H}]^+$, Na$^+$ formed by oxidation of complex 1 during the experiment.

### 2.1. IR spectroscopic studies

As previously described for related mixed-ligand $\text{V}^{IV}$-complexes that simultaneously include a salicylaldehyde semicarbazone and phenanthroline in the vanadium(IV) coordination sphere, N-acylhydrazone complexes 1 and 2 show quite complex spectra [32]. In particular, several bands corresponding to $\nu(C=O)$ and $\nu(C=N)$ in heterocyclic compounds lie in the 1650–1550 cm$^{-1}$ region [32,37,40]. Table 1 summarizes the tentative assignments made.

The non-observation of the $\nu(C=O)$ band, indicates the elimination of the amide functionality upon coordination to the $\text{V}^{IV}$-center. Instead, strong bands at $\sim 1620$–1635 cm$^{-1}$ are observed, which can be attributed to the asymmetric stretching vibration of the conjugated CH⋯N–N⋯C group, characteristic of the coordinated enolate form of the ligands [39,41]. These and other spectral changes such as those summarized in Table 1 are in agreement with LASSBio-1064 double deprotonation and tridentate coordination through the carbonyl oxygen (O$\text{CO}$), the hydrazone N atom (N$\text{H}$), and the phenolate oxygen (O$\text{ph}$) (Fig. 1) [42].

### 2.2. Characterization of the complexes in solution

Solutions of both compounds were studied by EPR and $^{51}$V NMR spectroscopies. For this purpose ca. 1 mM solutions of the complexes were prepared in DMF (and DMSO), kept at room temperature in contact with air and the change over time of their spectroscopic (EPR and $^{51}$V NMR) properties was studied.

Upon dissolving the complexes the solutions showed an orange-brown color. In DMF solution compound 1 showed very weak d–d bands in the visible range at ca. 580 and 700 nm. Compound 2 is more soluble in DMF than 1 and d–d bands were observed at ca. 800 nm (shoulder, $\epsilon = 49\text{ M}^{-1}\text{cm}^{-1}$), 707 nm ($\epsilon = 54\text{ M}^{-1}\text{cm}^{-1}$), ca. 500 nm (shoulder, $\epsilon = 92\text{ M}^{-1}\text{cm}^{-1}$) and a charge transfer band at 422 nm (yet $\epsilon = 1000\text{ M}^{-1}\text{cm}^{-1}$) which overlaps the band at 500 nm.

The EPR spectra of the complexes dissolved in DMF (and DMSO) were measured at 77 K. The spectra exhibit a hyperfine pattern typical of $\text{V}^{IV}$-complexes, consistent with the presence of monomeric $\text{V}^{IV}$-bound species with $d^4\text{xy}$ ground-state configuration. Fig. 2 shows the spectra of complex 1 in both solvents and Fig. 3 the variation of the EPR spectra of complex 2 (in DMF) with time.

Both complexes slowly oxidize. As expected, compound 2, containing phen as co-ligand, is significantly more stable toward oxidation and after 5 days it still showed the presence of a considerable amount of $\text{V}^{IV}$. Table 2 contains the spin Hamiltonian parameters obtained by simulation of the experimental spectra [45].

Once a particular binding mode is assumed, the values of $A_I$ can be estimated ($A_{II}^\text{est}$) using the additivity relationship proposed by Wüthrich [46] and Chasteen [47], with estimated accuracy of $\pm 3 \times 10^{-4} \text{ cm}^{-1}$. Assuming that O$\text{CO}$ binding contributes with $A_I(O\text{CO}) = 41.6 \times 10^{-4} \text{ cm}^{-1}$, taking an average value for $A_I(N\text{H}) = 41.6 \times 10^{-4} \text{ cm}^{-1}$ [48], $A_I(O_{\text{phenolate}} = O\text{CO}) = 38.9 \times 10^{-4} \text{ cm}^{-1}$, $A_I(N_{\text{phenanthroline}} = N_{\text{pyridine}}) = 40.4 \times 10^{-4} \text{ cm}^{-1}$ [52], $A_I(O\text{DMF}) = 43.7 \times 10^{-4} \text{ cm}^{-1}$ and $A_I(O_{\text{DMF}}) = 43 \times 10^{-4} \text{ cm}^{-1}$ [53] we obtained for both complexes the values for $A_{II}^\text{est}$ presented in Table 2. The $A_{II}^\text{est}$ agree well with the experimental values.

For complex 2 in DMF the parent N-acylhydrazone (LASSBio-1064) acts as a tridentate ligand binding with $O_{\text{phen}}, N_{\text{H}}, O_{\text{CO}}$ equatorial and the phenanthroline (phen) binds as a bidentate ligand through the two N donors, one N in the equatorial position and the other trans to the oxo oxygen donor (Fig. 1). This axial-equatorial

**Table 2**

<table>
<thead>
<tr>
<th>Complex</th>
<th>Solvent</th>
<th>$g_a, g_c$</th>
<th>$g_e$</th>
<th>$A_e, A_I$ ($\times 10^5$ cm$^{-1}$)</th>
<th>$A_e$ ($\times 10^6$ cm$^{-1}$)</th>
<th>$A_{II}^\text{est}$ ($\times 10^4$ cm$^{-1}$)$^a$</th>
<th>Proposed equatorial binding set$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DMF</td>
<td>1.980</td>
<td>1.950</td>
<td>56.9</td>
<td>165.4</td>
<td>165.8</td>
<td>$O_{\text{phen}}, O_{\text{CO}}, N_{\text{H}}$</td>
</tr>
<tr>
<td></td>
<td>DMSO</td>
<td>1.976</td>
<td>1.937</td>
<td>65.5</td>
<td>177.4</td>
<td>174.7</td>
<td>$O_{\text{water}} + O_{\text{DMF}}$</td>
</tr>
<tr>
<td>2</td>
<td>DMF</td>
<td>1.982</td>
<td>1.953</td>
<td>53.3</td>
<td>159.2</td>
<td>162.5</td>
<td>$O_{\text{phen}}, N_{\text{H}}, N_{\text{phen}}$</td>
</tr>
<tr>
<td></td>
<td>DMSO</td>
<td>$\approx$ 164</td>
<td></td>
<td></td>
<td></td>
<td>$O_{\text{phen}}, N_{\text{H}}, O_{\text{phen}}$</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ The following $A_e$ values were used in the calculation of $A_{II}^\text{est}$ (see text): $O_{\text{phenolate}} = 38.9 \times 10^{-4} \text{ cm}^{-1}$, $N_{\text{phen}} = 40.4 \times 10^{-4} \text{ cm}^{-1}$, $N_{\text{H}} = 41.6 \times 10^{-4} \text{ cm}^{-1}$, $O_{\text{CO}} = 43.7 \times 10^{-4} \text{ cm}^{-1}$, $O_{\text{DMF}} = 43 \times 10^{-4} \text{ cm}^{-1}$. For some of these their predicted contributions to the $A_e$ hyperfine coupling constant are not straightforward, namely the contributions of $N_{\text{H}}$ and of $O_{\text{DMF}}$. In fact, the $N_{\text{H}}$ is probably close to the values of imine N atoms and these may vary between 38.1 and 43.7 $\times 10^{-4} \text{ cm}^{-1}$ [44,48,49] while O$\text{CO}$ may vary between the contribution of a typical C–O carbonyl (43.7 $\times 10^{-4} \text{ cm}^{-1}$) [50], and of an O-enolate (37.6 $\times 10^{-4} \text{ cm}^{-1}$) [48], an average value in $[\text{V}^{IV}\text{(acac)}_2]$ being 41.6 $\times 10^{-4} \text{ cm}^{-1}$ [51].

$^b$ The $A_{II}^\text{est}$ does not change much if the bound solvent is DMF, H$_2$O or ethanol.

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Fig. 3. Changes observed with time (hours) in the central region of the first derivative EPR spectra for 1 mM solutions of complex 2 in DMF.

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The 51V NMR spectra measured at room temperature for the 1 mM solutions of the complexes in DMF confirm their progressive oxidation over time. One hour after dissolution of the complexes bands assignable to V^0 complexes could be already observed at ca. –540 ppm. The intensity of the bands increased with time and in the spectrum of complex 2 the resonances corresponding to vanadate oligomers (<10%) were found after 5 days [−553 ppm (V'_4) and −576 ppm (V'_5)]. We can, therefore, conclude that the same type of V^4- species is being formed upon oxidation of the two V^0O-complexes under air. Chemical shifts of V^0O2semicarbazone complexes have been reported and theoretically predicted in the −530 to −550 ppm range [58]. Thus the V^5- complexes formed probably correspond to species formulated as V^5O2(LASSBio1064)(solvent).

In DMSO immediately after dissolution some aggregation of the molecules is maintained and the EPR spectra at 77 K show some broadening of the lines and lower intensity of the spectra. This behavior has been previously observed [56,57]. For this complex the parameters obtained indicate significant solvolysis and substitution of the phenanthroline ligand by DMSO. Due to the low intensity of the spectra of complex 2 measured in DMSO it was not possible to carry out a good analysis of the spectra, but an estimate of the A2 values is given in Table 2. For complex 1 in DMSO, a solvent with good coordinating ability, the spectrum indicates almost total replacement of the N-acylhydrazone ligand by DMSO molecules, while in DMF the EPR spectra are consistent with the formula given in Fig. 1.

The 51V NMR spectra of LASSBio-1064 and its vanadium compounds 1 and 2 against promastigotes of Leishmania major.

In vitro activity of pentamidine (reference drug), LASSBio-1064 and its vanadium compounds 1 and 2 against promastigotes of Leishmania major.

Table 4

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 (μM)</th>
<th>Highest efficacy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentamidine</td>
<td>0.8 ± 0.2</td>
<td>72.6 ± 2.0**</td>
</tr>
<tr>
<td>LASSBio-1064</td>
<td>&gt;100</td>
<td>31.5 ± 5.9</td>
</tr>
<tr>
<td>1, [V^0O(LASSBio1064-2H)(H2O)]</td>
<td>&gt;100</td>
<td>26.6 ± 9.1</td>
</tr>
<tr>
<td>2, [V^0O(LASSBio1064-2H)(phen)]</td>
<td>22.1 ± 0.6</td>
<td>83.6 ± 6.0**</td>
</tr>
</tbody>
</table>

Data are reported as means ± S.E.M. Differences with a *p value <.01 were considered significant in relation of medium group and a **p value <.001 were considered significant in relation of vehicle group (DMSO).

2.3. Biological evaluation of the complexes

2.3.1. In vitro anti-T. cruzi activity

Both complexes and the NAH-ligand LASSBio-1064 were evaluated in vitro for their anti-T. cruzi activities against epimastigotes of Tulahuen 2 strain (Table 3). Results were compared to the reference drug Nifurtimox. All complexes were active in vitro against the epimastigote form of T. cruzi (Tulahuen 2 strain) showing IC50 values lower or of the same order of those of the reference drug Nifurtimox and the free ligand LASSBio1064.

2.3.2. In vitro anti-Leishmanial activity and toxicity to mammalian cells

To establish the leishmanicidal profile of LASSBio-1064 and its vanadium complexes (1 and 2), these were evaluated in vitro against promastigotes forms of L. major [60]. IC50 values obtained from these studies are shown in Table 4. Complex 2 exhibited antileishmanial activity (with the highest efficacy of 83.6 ± 6.0% and IC50 values of 22.1 ± 0.6 μM). However in molar units this V^5O-complex was less potent than pentamidine (with the highest efficacy of 72.6 ± 2.0% and IC50 values of 0.8 ± 0.2 μM). In contrast, LASSBio-1064 and complex 1 did not present activity against promastigotes forms of L. major (IC50 values > 100 μM).

Fig. 4 shows the cytotoxicity results for the LASSBio-1064 compounds and pentamidine (positive control) tested against murine macrophages using the MTT method [61]. The vanadium complex 1 at concentration of 100 μM and the vanadium complex 2 up to 30 μM showed deleterious activity on host cells evidenced by the trials of cell viability in murine macrophages. However, the LC50 values against peritoneal macrophages of complexes 1 and 2 are higher than 100 μM (Table 5). On the other hand, after 48 h of incubation, LASSBio-1064 and pentamidine did not affect the viability of inflammatory macrophages.
2.4. DNA interaction studies

To provide insight into the possible mechanism of antiparasitic action, the mixed-ligand compound \([\text{VIVO(LASSBio1064-2H)(phen)}] \cdot (\text{H}_2\text{O})\) was tested for its DNA interaction ability on plasmid DNA by atomic force microscopy (AFM) and on CT DNA by using DNA viscosity measurements and fluorescence spectroscopy. As previously described for structurally related oxidovanadium(IV) complexes the presence of phen in the coordination sphere would allow the intercalation between base pairs of the complex through this planar moiety \([32,37,38,62\text{e}64]\).

The image obtained by AFM after incubation of plasmid DNA with the complex for 24 h at 37 °C is depicted in Fig. 5, together with the image of the pBR322 plasmid alone incubated in the same conditions. Effects due to the interaction are clearly observed, such as kinks, crosslinking and supercoiling. Similar effects have been previously observed for metal complexes capable to intercalate DNA and, particularly, for other oxidovanadium(IV) complexes with DNA intercalating ligands \([32,37,38,62\text{e}64]\).

Upon interaction the complex increased the viscosity of CT DNA solution in a concentration dependent manner as depicted in Fig. 6. This type of behavior is usually attributed to intercalators as base pairs are separated to accommodate the binding ligand, leading to an increase of DNA length with a concomitant rise in viscosity \([65\text{e}67]\). Therefore, the results of the DNA viscosity measurements also suggest an intercalating interaction between compound 2 and DNA.

The fluorescence technique was not able to detect significant effects due to interaction of the complex with CT DNA. These studies showed only a very slight decrease of intensity, indicative of non efficient displacement of intercalated ethidium bromide by the mixed-ligand oxidovanadium(IV) compound (data not shown). Probably complex 2 binds DNA less tightly than ethidium bromide, therefore being unable to displace it.

2.5. Comments on the activity of the compounds

It is known that the biological activity of several metal based drugs is due to species with structures distinct from those characterized in the solid state. For example, the \(\text{V}^{\text{IV}}\)-compounds selected for clinical trials as potential insulin-enhancing drugs have demonstrated changes in the vanadium coordination sphere after dissolution, and particularly after being absorbed in the gastrointestinal tract \([36,68\text{e}70]\).

The present \(\text{V}^{\text{IV}}\)-compounds could act in biological media as produgs leading to \(\text{V}^{\text{III}}\)-and/or \(\text{V}^{\text{IV}}\)-LASSBio-1064 species that would be responsible of the observed activities. As the vanadium compounds show higher activity than the ligand and/or the metal salts, this is a clear indication that the activity is associated to...
vanadium species containing the ligand at least till the compounds are up-taken by cells. The higher activity of the metal compounds may be related to either an easier uptake and/or to their higher efficacy toward their targets. On the other hand, inside cells the vanadium speciation (not addressed in this work) may yield species containing or not the original ligands. The phenanthroline moiety may indeed be relevant in this respect; there are several other examples where phen and other related compounds, which may act as DNA intercalators, improve the biological activity of the metal-based systems [40,71].

3. Conclusions

Two new oxidovanadium(IV) complexes, \([\text{V}^\text{IV}(\text{LASSBio1064-2H})(\text{H}_2\text{O})]\) and \([\text{V}^\text{IV}(\text{LASSBio1064-2H})(\text{phen})](\text{H}_2\text{O})\), including the N-acylhydrazone LASSBio-1064 ligand, were synthesized and characterized in the solid state and in solution. Both complexes and the N-acylhydrazone compound showed trypanosomicidal activity on \(T. cruzi\), the etiological agent of Chagas disease, slightly higher (in a molar basis) than that of the trypanocidal drug Nifurtimox. However, only the mixed-ligand vanadyl complex 2 containing phen as co-ligand showed activity on \(T. major\) promastigotes.

DNA interaction studies showed that this mixed-ligand complex is able to interact with the biomolecule probably through an intercalative mode, pointing out at DNA as a probable target. Other potential targets could not be discarded. A higher uptake by cells and/or the interaction with DNA could be responsible for the increased antileishmanial activity of \([\text{V}^\text{IV}(\text{LASSBio1064-2H})(\text{phen})](\text{H}_2\text{O})\) as compared with \([\text{V}^\text{IV}(\text{LASSBio1064-2H})(\text{H}_2\text{O})]\) and LASSBio1064. Both \(\text{V}^\text{IV}\)-compounds showed low toxicity on mammalian cells and, therefore, a good selectivity toward the parasite.

The results suggest that \([\text{V}^\text{IV}(\text{LASSBio1064-2H})(\text{phen})](\text{H}_2\text{O})\) may be a promising compound for further drug development stages. Moreover, further speciation studies would help in establishing the actual species responsible of the detected biological activity.

4. Materials and methods

4.1. Materials

All common laboratory chemicals, including \([\text{V}^\text{IV}(\text{acac})_2]\), where \(\text{acac} = \text{acetylacetonate, 1,10-phenanthroline (phen), VOSO}_4\cdot \text{SH}_2\text{O},\) pentamidine, Schneider’s milieu, Fetal Bovine Serum and Dubbeco’s Modified Eagle’s milieu (DMEM), were from commercial sources and were used without further purification. LASSBio-1064, \((E)-N'$-\) (2-hydroxybenzylidine-4-chlorobenzohydrazide), was synthesized and characterized as previously described by reaction of salicylaldehyde (2-hydroxy-benzaldehyde) and 4-chlorobenzohydrazide in 1:1 M ratio in ethanol—HCl milieu [72–74].

4.2. Synthesis of \([\text{V}^\text{IV}(\text{LASSBio1064-2H})(\text{H}_2\text{O})]\), 1

LASSBio-1064 (50 mg, 0.188 mmol) and \(\text{VOSO}_4\cdot \text{SH}_2\text{O}\) (47 mg, 0.188 mmol) were suspended in 20 mL of absolute ethanol previously purged with nitrogen for 10 min. The suspension was heated at reflux under nitrogen for 24 h and the reddish-brown solution obtained was partially evaporated (final volume 3 mL). The brown solid formed was filtered off from the mixture and washed with 2 mL portions of diethyl ether, acetone and methanol. Yield: 40% (27 mg). Anal. % calc. for \(\text{C}_{21}\text{H}_{21}\text{ClN}_{2}\text{O}_5\): C, 47.02; H, 3.10; N, 7.83. Found: C, 46.79; H, 3.34; N, 7.78. ESI-MS (MeOH) \(m/z\) Found = 579.3 (100%) \([\text{V}^\text{IV}(\text{LASSBio1064-2H})(\text{H}_2\text{O})]\) \(\text{Na}^+\), with \(m/z = 380.6\) does not fit). Probably the compound oxidized in this dilute solution and what is detected is the corresponding \(\text{V}^\text{IV}\)-complex \([(\text{V}^\text{IV}(\text{LASSBio1064-4H}))(\text{Na}^+)\) for which the molecular weight is 378.62 and the \(m/z\) for the molecular ion is 379.6, which fits well with the experimental.

4.3. Synthesis of the mixed-ligand vanadyl complex \([\text{V}^\text{IV}(\text{LASSBio1064-2H})(\text{phen})](\text{H}_2\text{O})\), 2

LASSBio-1064 (50 mg, 0.188 mmol), 1,10-phenanthroline (34 mg, 0.188 mmol) and \([\text{V}^\text{IV}(\text{acac})_2]\) (50 mg, 0.188 mmol) were suspended in 20 mL of absolute ethanol previously purged with nitrogen for 10 min. Nitrogen was kept passing through the solution for ca. 10 min. The suspension was heated at reflux under nitrogen for 24 h and the reddish-brown solid formed was filtered off from the hot mixture, and was washed three times with 2 mL portions of ethanol:diethyl ether (1:1). Yield: 69% (70 mg). Anal. % calc. for \(\text{C}_{26}\text{H}_{27}\text{ClN}_{2}\text{O}_5\cdot \text{H}_2\text{O}\): C, 58.06; H, 3.56; N, 10.42. Found: C, 57.85; H, 3.53; N, 10.78. ESI-MS (MeOH) \(m/z\) [Found (calc.): 520.2 (520.0) (40%) \([\text{M} + \text{H}^+]\)]

4.4. Physicochemical characterization

C, H and N analyses were carried out with a Carlo Erba Model EA1108 elemental analyzer. Conductimetric measurements were carried out at 25 °C in 10⁻³ M dimethylformamide (DMF) solutions using a Conductivity Meter 4310 Jenway [75]. A 500-MS Varian Ion Trap Mass Spectrometer was used to measure electrospray ionization mass spectra (ESI-MS) of methanol solutions of the complexes in the positive mode; the complexes were first dissolved in a minimum amount of DMF and then diluted with MeOH. Each spectrum was obtained as a combination of several scans for each sample. FTIR spectra (4000–400 cm⁻¹) of the complexes and the free ligand were measured as KBr pellets with a Bomen FTIR model MB102 instrument. The UV–Vis absorption spectra were measured with a Perkin Elmer Lambda 35 spectrophotometer and ⁵¹V NMR spectra of ca. 1 mM solutions of the complexes in DMSO and DMF (p.a. grade) were recorded on a Bruker Avance III 400 MHz instrument: (i) after dissolution, and (ii) during a 5 day period standing in aerobic conditions at room temperature. ⁵¹V chemical shifts were referenced relative to neat VOCl₃ as external standard. EPR spectra were recorded at 77 K with a Bruker ESP 300E X-band spectrometer coupled to a Bruker ER04I X-band frequency meter (9.45 GHz). The complexes were dissolved at room temperature in DMSO or DMF (p.a. grade), previously degassed with N₂ for 10 min, using ultrasound to assist the dissolution of the solid, and the obtained solutions were then frozen in liquid nitrogen. The spin Hamiltonian parameters were obtained by simulation of the spectra with the computer program of Rockenbauer and Korecz [76]. Stability of the complexes in 6% DMSO–H₂O·10⁻² M solution at room temperature was followed by conductivity measurements for 5 days.

4.5. In vitro anti-\(T. cruzi\) activity

\(T. cruzi\) epimastigotes (Tulahuan 2 strain) were grown at 28 °C in an axenic milieu (BHI-Tryptose), as previously described, complemented with 5% fetal calf serum [28,29,77]. Cells were harvested in the late log phase, re-suspended in fresh milieu, counted in Neubauer’s chamber and placed in 24-well plates (2 × 10⁶/mL). Cell growth was measured as the absorbance of the culture at 590 nm, which was proved to be proportional to the number of cells. Before inoculation the media were supplemented with the indicated amount of the studied compound from a stock solution in DMSO prepared immediately before use. The final concentration of DMSO in the culture media never exceeded 1% and the control was run in the presence of 1% DMSO as well as in the absence of any compound. No effect on epimastigotes growth was observed by the presence of up to 1% DMSO in the culture media. Nifurtimox was used as the reference trypanosomicidal drug. The percentage of growth inhibition was calculated as follows [1 − ([\text{Ap} − \text{Ap0}]/[\text{Ac} − \text{Ap0}]) × 100, where \(\text{Ap} = A_{590}\) of the culture containing the studied compound at
day 5: $A_{0p} = A_{500}$ of the culture containing the studied compound right after addition of the inocula (day 0); $Ac = A_{500}$ of the culture in the absence of any compound (control) at day 5; $A_{0C} = A_{500}$ in the absence of the compound at day 0. To determine IC_{50} values parasite growth was followed in the absence (control) and presence of increasing concentrations of the corresponding compound. The IC_{50} values were determined as the drug concentrations required to reduce by half the absorbance of that of the control (without compound) [28,29,77].

4.6. In vitro activity against L. major

Promastigotes of L. major IOCl0581 (MHOM/SU/1973/5-ASKH) were obtained from Leishmania collection of the Oswaldo Cruz Institute. The parasites were maintained in vitro in Schneider’s milieu, supplemented with 10% FBS and 2% human urine. Stock solutions of LASSBio-1064, [V^6/0(LASSBio1064-2H)(H_2O)], 1, [V^6/0(LASSBio1064-2H)(phen)] (H_2O), 2, and pentamidine (reference leishmanicidal drug) were prepared in DMSO immediately before use. The cytotoxicity of LASSBio1064, complexes 1 and 2 and pentamidine against promastigotes was then determined. Stationary phase L. major promastigotes were plated in 96-well vessels (Nunc) at 1 x 10^6 cells per well, in Schneider’s milieu, supplemented with 10% FBS and 2% human urine, and increasing concentrations (0.3 μM—100 μM) of each compound solution were added. Cells were also cultured in a milieu free of compounds or vehicle (basal growth control) or with DMSO 0.1% (vehicle control). After 48 h extracellular load of L. major promastigotes was estimated by counting the promastigotes in Schneider’s milieu in a CELM automatic cell counter (model CC530) [60].

4.7. Cytotoxicity against host cells

Inflammatory macrophages were obtained using adult Swiss mice weighing 20–30 g each, males or females, of about 6–8 weeks of age. All animals came from the breeding unit of the BIOCEN—UFAL. The animals were maintained with free access to food and water and kept at 25–28 °C under a controlled 12 h light/dark cycle. All animals used in this work were treated according to the regulations established by the Ethics Committee of Federal University of Alagoas—UFAL (number: 23065.024392/2009-90) for animal handling. To evaluate the cytotoxic activity against the murine macrophages the animals were injected with 1.0 mL of thioglycolate 4%. After 4 days the animals were killed by cervical dislocation. The peritoneal cavity was washed with 5.0 mL of Hank’s buffer salt solution (HBSS, Sigma), and after gentle manual massage the exudate was retrieved to obtain inflammatory macrophages. The peritoneal macrophages were plated in 96-well vessels at 2 x 10^5 cells per well in complete culture milieu 10% FBS at 37 °C. After 1 h wells were washed with warm HBSS to remove non-adherent cells, leaving approximately 1 x 10^5 adherent macrophages. All cultures were done in DMEM complete supplemented with 10% FBS. The compounds and pentamidine were added at serial concentrations (0.3–100 μM). The cells were also cultured with milieu free from compounds or vehicle (basal growth control) or in media with DMSO 0.1% (vehicle control). Positive control (dead cells) was obtained by cellular lysis with 1% of Triton 100× in DMEM complete. After 48 h, the cytotoxicity was evaluated by the MTT assay [61].

4.7.1. Statistical analysis

Data obtained from in vitro experiments were expressed as the mean ± standard error of the mean (Mean ± S.E.M.) of triplicate cultures of representative assays. Statistical differences between the treated and the control groups were evaluated by ANOVA and Dunnett hoc tests. Differences with a p value < 0.05 or lower were considered significant.

4.8. DNA interaction studies

4.8.1. Atomic force microscopy (AFM) studies

To optimize the observation of the conformational changes in the tertiary structure of pBR322 plasmid DNA, the plasmid was heated at 60 °C for 30 min to obtain mostly the open circular form. Then 15 ng of pBR322 DNA were incubated in an appropriate volume with the required compound concentration corresponding to the molar ratio base pairs (bp):compound 5:1. The oxidovanadium(IV) complex was dissolved in a minimal amount of DMSO and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (HEPES) pH 7.4 was then added up to the required concentration. All the solutions as well as Milli-Q® water were filtered with 0.2 μm PP030/3 filters (Schleicher & Schuell GmbH, Germany). Immediately after preparing the solutions incubations were carried out at 37 °C for 24 h. Samples were prepared by placing a drop of DNA solution or DNA-compound solution onto mica (TED PELLA, INC. California, USA). After adsorption for 5 min at room temperature, the samples were rinsed for 10 s in a jet of deionized water (18 MΩ cm^-1 from a Milli-Q® water purification system) directed onto the mica surface, blow dried with compressed argon and then imaged by AFM.

The samples were imaged by a Nanoscope III Multimode AFM (Digital Instruments Inc., Santa Barbara, CA) operating in tapping mode in air at a scan rate of 1–3 Hz. The AFM probe was 125 mm-long monocrystalline silicon cantilever with integrated conical shaped Si tips (Nanosensors GmbH Germany) with an average resonance frequency f_{res} = 330 kHz and spring constant K = 50 N/m. The cantilever was rectangular and the tip radius given by the supplier was 10 nm, a cone angle of 35° and high aspect ratio. The images were obtained at room temperature (t = 23 ± 2 °C) and the relative humidity was usually lower than 40% [32,38].

4.8.2. Fluorescence studies

To a 50 μM calf thymus DNA (CT DNA) solution in Milli-Q® water, 30 μL of a 5 mM ethidium bromide solution was added to get a 1:1 M ratio of ethidium bromide to DNA. The mixture was incubated for 30 min at 37 °C. Increasing amounts of a 1.5 mM DMSO/Milli-Q® water stock solution of the complex under study were added to reach the following final concentrations of the complex: 0, 10, 20, 30, 40 and 50 μM. Fluorescence spectra (λ_{em} = 520 nm) were recorded at room temperature with a HORIBA BA Nanolog iHR 320 spectrophotometer in the wavelength range 530–670 nm after a short incubation time [48].

4.8.3. Viscosity measurements

Viscosity experiments were conducted at 25 °C on an automated and viscometer model SV-10. Stock solutions of each complex were prepared in DMSO/water. A 1 mM CT DNA solution was diluted 1:4 with TE buffer. For each complex increasing amounts of complex stock solution were added to the DNA solution to reach complex/DNA molar ratios in the range 0–20. The DMSO amount in the samples never exceeded 2%. After thermal equilibrium was achieved (15 min), the viscosity of each sample was repeatedly measured. Mean values of five measurements carried out at intervals of 1 min were used to obtain the viscosity of each sample [48].

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