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# Antiparasitic Activity of Oxindolimine–Metal Complexes against Chagas Disease

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**Abstract:** Some copper(II) and zinc(II) complexes with oxindolimine ligands were tested regarding their trypanocidal properties. These complexes have already shown good biological activity in the inhibition of tumor cell proliferation, having DNA and mitochondria as main targets, through an oxidative mechanism, and inducing apoptosis. Herein, we demonstrate that they also have significant activity against the infective trypomastigote forms and the intracellular amastigote forms of *T. cruzi*, modulated by the metal ion as well as by the oxindolimine ligand. Selective indexes ($LC_{50}/IC_{50}$) determined for both zinc(II) and copper(II) complexes, are higher after 24 or 48 h incubation with trypomastigotes, in comparison to traditional drugs used in clinics, such as benznidazole, and other metal-based compounds previously reported in the literature. Additionally, tests against amastigotes indicated infection index <10% (% of infected macrophages/average number of amastigotes per macrophage), after 24 or 48 h in the presence of zinc(II) (60–80 μM) or analogous copper(II) complexes (10–25 μM). The copper complexes exhibit further oxidative properties, being able to damage DNA, proteins and carbohydrates, in the presence of hydrogen peroxide, with the generation of hydroxyl radicals. This redox reactivity could explain its better performance towards the parasites in relation to the zinc analogs. However, both copper and zinc complexes display good selective indexes, indicating that the influence of the ligand is also crucial, and is probably related to the inhibition of some crucial proteins.

**Keywords:** oxindolimine ligands; metal complexes; Chagas disease; *T. cruzi*; trypanocidal activity; mechanism of action



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## 1. Introduction

Chagas disease, also known as American trypanosomiasis, is among the potentially fatal neglected tropical diseases. According to World Health Organization (WHO) data [1], it afflicts more than 6 million persons in extremely poor areas worldwide, especially in Latin America, where it is present in 20 countries, with an estimated 70 million people at risk of infection [2]. This disease was named after Carlos Ribeiro Justiniano Chagas, a Brazilian physician and researcher who discovered it in 1909. Further, Chagas disease has been described in non-endemic areas due to migration [3,4], and so it has become a public health issue even in developed nations [5]. Cure is only possible if treatment is administered soon after infection; thus, exposure to the parasite mostly leads to chronic infection. The reasons for this picture involve different factors, such as insufficient knowledge of the disease, a deficient market that makes the development of new drugs or new treatments not financially attractive for the pharmaceutical industry [6], and inadequate or non-existing

efficient drugs once infection is established. Some recent reviews report efforts to elucidate the fundamental molecular and cell biology of parasitic trypanosomatids as well as the diseases they can cause [7,8].

The parasite responsible for this disease is the flagellate protozoa *Trypanosoma cruzi*, injected in the blood of human beings by triatominae bugs, in its trypomastigote form [9]. Transported through the blood stream, the parasites can enter host cells, including macrophages, where they are transformed into their amastigote forms. A recent review focused on the *T. cruzi* life cycle [10], providing a comprehensive update on its morphological forms and genetic diversity, aiming at identifying intervention points to cure the disease.

Current clinical treatments of Chagas disease are based on drugs that have been developed many decades ago, such as benznidazole (N-benzyl-2-nitroimidazole-1-acetamide) or nifurtimox (4[(5-nitrofurfurylidene)amino]-3-methylthiomorpholine-1,1-dioxide). Although active in earlier phases of the disease, these drugs show severe toxic side effects, and are inactive in the chronic phase of infection [11]. Therefore, many efforts have been made by several research groups in developing new, more efficient, and less toxic, usually organic compounds [12,13]. A wide range of compounds have been developed, with quite different structural features, and some of them have been clinically tested against trypanosomiases [14]. Among these chemotherapeutic agents there are natural as well as synthetic compounds, including naphthoquinones [15,16], alkaloids, antihistaminics, antibiotics, thiazolidinones [17], aminoquinolines, and thiazoles [18]. More recently, leucinostatins, natural products derived from fungi collected from soil samples, showed potent activity against intracellular and replicative amastigote forms of the parasite, with no host cell toxicity up to 1.5 μM [19]. On the other hand, biological studies on macrophage-derived peroxynitrite ($ONOO^-$ and ONOOH) formation, a strong oxidant arising from the reaction of nitric oxide ($^{\cdot}NO$) with superoxide radical ($O_2^{\cdot -}$), revealed that $^{\cdot}NO$ plays a central role in the control of acute infection by *T. cruzi* [20]. When those reactive species are formed simultaneously, the generation of peroxynitrite leads to severe cellular oxidative damage and morphological disturbance in internalized parasites [21].

Among synthetic compounds, different metal complexes with diverse ligands have been reported as showing significant trypanocidal activity. Some ternary nickel(II) complexes with imine and azapurine derivatives [22] showed high antitrypanosomatid activity against the epimastigote, amastigote, and trypomastigote forms of the parasite, after 72 h of culture, with $IC_{50}$ in the 1–90 μM range, lower than those of the reference drug, benznidazole (BZ). A gold(III) complex with tridentate thiosemicarbazone ligands coordinated by an *ONS* donor set, [AuCl($L^{Me}$)] was found to be more active and more selective than its precursor ligand and the standard drug benznidazole with a selective index SI (trypomastigote/amastigote) higher than 200 [23]. Some of these complexes, however, despite showing a suppressive effect on the parasitemia, were not curative, since there are several reasons contributing to the incurability of the disease.

Therefore, the list of complexes tested in the development of new antiparasitic drugs against trypanosomiasis includes Co(II), or Cu(II) with triazole derivatives [24], Pt(II) or Pd(II) with thiosemicarbazones [25], Ru(II) with lapachol [26,27] or thiosemicarbazones [28], and vanadium with polypyridyl ligands [29]. Those studies are based on the action of such complexes toward different targets, such as cysteine proteases [30], hypoxanthine−guanine phosphoribosyl-transferases (HGPRTs) [31], and DNA [32].

Some recent studies reported the survival of *T. cruzi* exposed to benznidazole (BZ), using genetically modified parasites that overexpress different DNA repair proteins [33]. These investigations indicated that this drug causes double-stranded DNA breaks in the parasite, reinforcing its mechanism of action by reactive oxygen species (ROS) formation, particularly hydroxyl radical [34]. The importance of ROS in *T. cruzi* infections has been emphasized by showing that high levels of ROS are deleterious to the parasite. However, when ROS production was inhibited in the host cell, a significant reduced proliferation of wild-type parasites was also reported [35]. Further, overexpression of mitochondrial DNA repair proteins increases parasite survival upon exposure to benznidazole, indicating that

mitochondrial DNA is also a target. More recent studies [36] described an intimate relation between the parasite and the host protein U2AF35 that binds to RNA at the polypyrimidine tract [37], and is essential for initiating the RNA processing, significantly affecting the host cell in their functions.

Since our group has developed some oxindolimine–metal complexes capable of generating ROS and showing significant antitumor properties [38], based on oxidative damage to DNA and mitochondria, besides their inhibition of selected proteins, we decided to test them as potential antiparasitic agents. Previously, some oxindolimine-copper, zinc, and vanadyl complexes were tested against *Schistosoma mansoni* worms. Copper(II) complexes showed 50% inhibitory concentrations of 30 to 45 μM, and demonstrated greater antischistosomal properties than the analogous zinc and vanadyl complexes regarding lethality, reduction in motor activity, and oviposition [39]. Analogous zinc complexes were active after 72 h treatment, and vanadyl complexes were inactive up to 500 μM, even if they quite inhibited oviposition. Results showed that both copper and zinc easily cross the cell membrane and induce severe tegumental damage in schistosomes.

Herein, the reactivity of four such oxindolimine compounds, metalated with copper(II) or zinc(II), against the trypomastigote and amastigote forms of *T. cruzi* is reported.

## 2. Experimental Section

### *2.1. Synthesis of the Ligands*

Two different oxindolimine ligands were previously prepared by condensation reaction of 2,3-dioxindole with 1,3-diaminopropane (isapn), or 2-(2-aminoethyl) pyridine (isaepy). *Briefly*, 1.47 g isatin (10 mmol) was dissolved in 40 mL ethanol, in a 125 mL flask. To this solution, 420 μL (5 mmol) 1,3-diaminopropane was added, adjusting the final pH to 5 with a few drops of 0.1 mol/L HCl solution. The reaction solution was maintained under stirring for 6 h, until yellow crystals of the isapn ligand were formed. The precipitate was filtered, washed with cold ethanol and ethyl ether, and stored in a desiccator under reduced pressure. Yield: 79%. The other ligand isaepy was analogously prepared; yield 72%. Analytical data: for isapn, yellow powder, 68.66%C, 4.85 %H, 16.86 %N; Calc. for $C_{19}H_{16}N_4O_2$, 67.64%C, 4.72 %H, 16.52 %N; MS (ESI+): $m/z$ = 333.1, [M + 1]+ in $CH_3OH$, MW = 332.36 g/mol for $C_{19}H_{16}N_4O_2$; for *isaepy*, yellow powder, 71.70%C, 5.21 %H, 16.72 %N; Calc. for $C_{15}H_{13}N_3O$, 71.17%C, 5.32 %H, 16.45 %N.

### *2.2. Syntheses of the Metal Complexes*

The corresponding metal complexes, $[Cu(isapn)](ClO_4)_2$ **1**, $[Zn(isapn)]ClO_4$ **2** $[Cu(isaepy)H_2O]ClO_4$ **3**, $[Zn(isaepy)Cl_2]$ **4**, and $[Cu(isaepy)_2](ClO_4)_2$ **5** (shown in Figure 1), have been prepared by metalation in situ of these ligands with suitable metal salts, as reported in previous studies [40,41]. According to the pH adjusted at the metalation step, the keto or the enol form was preferentially obtained, although in a solution at pH 7.4 both forms are detected, as indicated by mass spectrometry measurements. The crystals formed were filtered and washed with a few mL ethanol and ethyl ether, and afterwards dried under suction. The corresponding products (yields in the range of 65 to 90%) were stored in a desiccator under reduced pressure. They were identified by UV/Vis, IR spectroscopies, and mass spectrometry, in addition to elemental analyses. The copper(II) complexes were further characterized using EPR, and the analogous zinc(II) using NMR spectroscopy. Analytical data: Complex **1** $[Cu(isapn)](ClO_4)_2$, brown crystals, 85% yield, MW 594.80 g/mol. Experim. data: 39.33%C, 2.88%H, 9.69%N, 10.58%Cu; Calc. for $C_{19}H_{16}N_4O_{10}Cl_2Cu$, 38.36%C, 2.71 %H, 9.42%N, 10.72%Cu; MS (ESI+): $m/z$ found: 395.02 (calcd.: 395.07, for $C_{19}H_{16}N_4O_2Cu$). Complex **2** $[Zn(isapn)](ClO_4)$, orange solid, 85% yield—Experim. data: 45.71%C, 3.48%H, 11.31%N; Calc. for $C_{19}H_{15}N_4O_6ClZn$, 45.99%C, 3.05%H, 11.29%N; MS (ESI+) at pH 7: $m/z$ = 396.9, in $CH_3OH$–$H_2O$, MW = 396.05, fragment monocation $[C_{19}H_{16}N_4O_2\ ^{64}Zn]$; 398.9 [MW = 398.05 for the isotopic pattern of the ($^{66}$Zn) monocation] [38]. Complex **3** $[Cu(isaepy)(H_2O)]ClO_4$, Yield 66%, MW 449.31 g/mol. Experim. data: 40.10%C, 3.59%H, 9.35%N; Calc. for $C_{15}H_{16}N_3O_7ClCu$, 40.73%C, 3.41 %H,

9.18%N. MS (ESI+): $m/z$ = 314.1 [MW = 431.29, in $CH_3OH/H_2O$, fragment $C_{15}H_{12}N_3OCu$]; 316.1 [isotopic pattern ($Cu^{63/65}$) monocation]; 564.1 [keto-form, fragment $C_{30}H_{24}N_6O_2Cu$ (compound **5**, MW = 566.11)]; 566.2 [isotopic pattern ($Cu^{63/65}$) monocation]. Complex **4** [Zn(isaepy)$Cl_2$], orange solid, yield 90%, MW 387.56 g/mol. Experim. 46.49%C, 3.38%H, 10.84%N; Calc. for $C_{15}H_{13}N_3OCl_2Zn$, 46.45%C, 3.41%H, 11.01%N. MS ($ESI^+$): $m/z$ = 350.0 MW = 387.58, in $CH_3OH/H_2O$, monocation fragment $[M+1]^+$; $[Zn(isaepy)(H_2O)]^+ \cdot H_2O$, $[C_{15}H_{16}N_3O_3{}^{64}Zn]$, and 352.0 [MW = 352.04 g/mol $[C_{15}H_{16}N_3O_3{}^{66}Zn]$. Complex **5** $[Cu(isaepy)_2](ClO_4)_2 \cdot 2\ H_2O$, brown crystals, yield 86%, MW 765.02 g. Experim. C, 45.69; H, 3.52; N, 10.08%. Calc. for $C_{30}H_{26}N_6O_2Cu(ClO_4)_2 \cdot 2H_2O$: C, 44.98%C; H, 3.76 %H; 10.45 %N. MS (ESI+): $m/z$ = 565.12 [MW = 565.15, in $CH_3CN$, fragment $C_{30}H_{26}N_6O_2Cu$]; 563.12 [isotopic pattern ($Cu^{63/65}$) monocation]; 312.02 [MW = 312.81, in $CH_3CN$, fragment $C_{15}H_{11}N_3OCu$] [42].

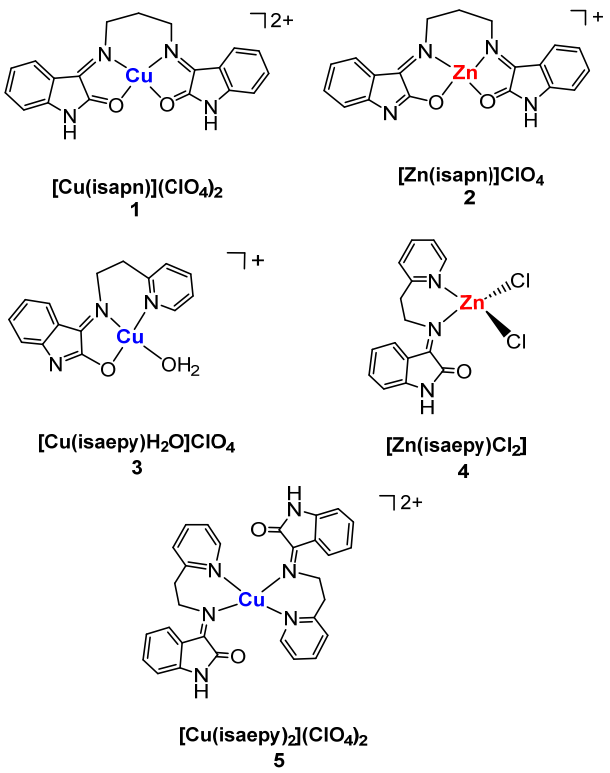


**Figure 1.** Structures of the oxindolimine–metal complexes, as isolated in solid state.

### *2.3. Materials and Methods*

Most of the reagents used in the syntheses of the metal complexes were purchased from Merck or Sigma-Aldrich Co. Elemental analyses using a 2400 CNH Elemental Analyzer (Perkin-Elmer, Billerica, MA, USA), or metal analyses (ICP-OES, Spectro Arcos, Spectro/AMETEK, Kleve, Germany), and NMR spectra, using a DRX-500 instrument (from Bruker, Karlsruhe, Germany), operating at 500 MHz, were performed at the *Central Analítica* of our Institution (Facility Center, https://www.iq.usp.br/portaliqusp/?q=en/services/analytical-center, accessed on 29 September 2023). IR spectra were recorded in a BOMEM 3.0 (diffuse reflectance) instrument (Quebec, QC, Canada), in the range of 4000–400 $cm^{-1}$, while UV/Vis spectra were recorded in an UV-1650PC equipment from Shimadzu (Kyoto, Japan). EPR spectra were registered using an EMX spectrometer from Bruker Instruments (Karlsruhe, Germany), operating at X-band (9.5 GHz), 100 kHz modulation frequency, and 20.0 mW power, using standard Wilmad quartz tubes and quartz Dewar (Vineland, NJ, USA). DPPH (α, α′–diphenyl-β-picrylhydrazyl) was used as the frequency calibrant (g = 2.0036), with samples as frozen $CH_3OH$ or $CH_3CH_2OH/H_2O$ (4:1) solutions, at 77 K. A modulation amplitude of 15 G, and $3.56 \times 10^2$ receiver gain were usually employed. Sim-

ulation and analyses of spectra were provided by the EasySpin 5.2.35 software package [42], in a MatLab environment.

### *2.4. Cells and Parasites*

The studied *T. cruzi* parasites were from Y strain, classified as TcII among the six discrete typing unit (DTU) groups [43]. Mouse peritoneal macrophages were used in MTT assays. Biological tests of the viability of different forms of the parasites, in the presence of the metal complexes or the free ligands, were carried out by using a Neubauer chamber or by MTT assays, as described next. All experiments were carried out in triplicate. Original LLC-MK2 cell lines (Macaca mulatta, code 0146, purchased from Banco de Células do Rio de Janeiro, RJ, Brazil—https://bcrj.org.br/accessed on 29 September 2023) in DME medium, supplemented by 10% fetal bovine serum at 37 °C, and 5% $CO_2$, were used as control and as cells to be infected by the parasites. Graphical treatments and statistical analyses were performed with the GraphPad Prism version 5.0 or 8.0.

### *2.5. Viability Test in Mouse Peritoneal Macrophages (MTT Assay)*

C57BL/6 mice were stimulated with 3% thioglycolate, three days before obtaining the peritoneal macrophages. To verify the effect of the compounds on the viability of macrophages, an assay with MTT [3-(4,5-dimethyl-2-thiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] was performed [44,45]. A total of $2 \times 10^5$ cells were plated in each well of a 96-well culture plate, and after adherence of the cells, 200 µL of DME (Dulbecco's modified Eagle's) medium, supplemented with 10% fetal bovine serum, was added, containing different concentrations of the compounds. Then, the culture plate was incubated for a period of 24 or 48 h in an oven at 37 °C, with 5% $CO_2$. Subsequently, 22 µL of MTT (5 mg/mL) was added to each well and the plate was incubated for an additional 4 h. After this incubation period, the supernatant was removed from each well and 80 µL of DMSO was added; 5 min later, the absorbance of each well in the plate was measured in a spectrophotometer at 492 nm. The protocol was approved by the UFMG ethical committee (CEUA 2/2018).

### *2.6. Effect of Compounds on the Trypomastigote Forms of T. cruzi*

Trypomastigotes were obtained by infection of LLC-MK2 cell lines (*Macaca mulatta*) in DME medium, that were also used as control, supplemented with 10% fetal bovine serum at 37 °C, and 5% $CO_2$, as previously described [46]. Five days after infection, trypomastigotes released into the medium were collected, washed with PBS by centrifugation, pelleted at 10,000× rpm for 10 min, and re-suspended to adequate cell density of the experiment in DME medium supplemented with 10% fetal bovine serum at 37 °C for 24 or 48 h, in the presence of aliquots of different concentrations of each copper or zinc compound, solubilized in aqueous solution containing 1% DMSO. To each well of a 96-well culture plate, 100 µL of culture medium containing $2 \times 10^5$ parasites in the trypomastigote form and 100 µL of the complex solutions in different concentrations were added. After an incubation period of 24 or 48 h, the viability of *T. cruzi* trypomastigotes was assessed by verifying the mobility of the parasites using optical microscopy, through counting in a Neubauer chamber.

### *2.7. Evaluation of the Effect of Compounds on Amastigote Forms of T. cruzi*

To perform this test, $5 \times 10^5$ peritoneal macrophages were plated on coverslips in 24-well plates and incubated at 37 °C in a 5% $CO_2$ oven for 2 h. Subsequently, *T. cruzi* trypomastigotes were added in a 5: 1 ratio (parasite/macrophage) and the cultures were incubated for another 2 h. After incubation, the wells were washed three times with RPMI medium, and supplemented with 10% fetal bovine serum at 37 °C to remove free parasites. Then, 1 mL of different concentrations of the compounds of copper (10–25 µmol/L) and zinc (60–155 µmol/L) in 1% DMSO aqueous solution was added, and the plate incubated for 24 h or 48 h. We also used benznidazole as a control in our tests. After the incubation period, the coverslips were fixed, using the fast panoptic staining kit, for later verification

of the inhibitory activity of the compounds against the parasite. To determine the inhibitory activity, the amastigote-infected macrophages and uninfected macrophages were counted, totaling 300 macrophages per count.

### *2.8. MTT Assay with Trypomastigote Parasites*

*T. cruzi* trypomastigotes, Y strain, were maintained by infection in LLC-MK2 cells as described above (item 2.6). Approximately $1 \times 10^7$ trypomastigotes were added to 16 wells of a 24-well plate, to which the compounds studied were added at concentrations ranging from 10 to 100 µM at first, and 1 to 10 µM depending on the results of the initial screening. Two of the sixteen wells containing trypomastigotes were used as control for the DMSO concentration at 1%, used for the solubilization of the compounds, and two other wells were used as control for the trypomastigotes' viability.

The trypomastigotes were incubated at 37 °C and 5% $CO_2$. After 24 h incubation, biological activity was measured using a colorimetric MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; 2.5 mg/mL). Readings were conducted in a Tecan Infinite F200 microplate reader at a wavelength of 565 nm. Assays were performed at least in triplicate. The absorbance values of wells containing only medium and reagents were used as blank for this assay.

## 3. Results and Discussion

### *3.1. Characterization and Stability of the Metal Complexes*

The oxindolimine complexes investigated in our studies are very stable, and can be isolated in different tautomeric forms, depending on the pH adjusted during the metalation step in their syntheses (see Figure 2). Both species, the keto and the enol forms, co-exist in solution, depending on the pH, as detected by EPR spectra in the case of copper(II) species [40], or NMR spectra for the zinc(II) analogs [41], corroborated by ESI-MS data.

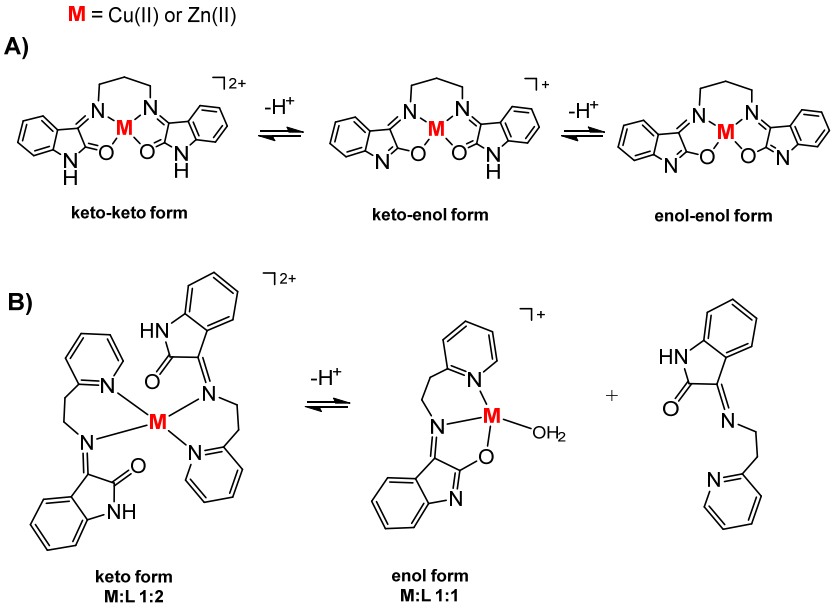


**Figure 2.** Tautomeric equilibria, depending on the pH, verified with copper(II) or zinc(II) oxindolimine complexes: (**A**) with the *isapn* ligand, and (**B**) with the *isaepy* ligand.

In the case of the copper complexes **1**, **3**, and **5**, the corresponding EPR hyperfine structure parameters (see Table 1) have been already reported [47], showing that the determined values for the $g_{//}/A_{//}$ ratio are much lower for the enol forms (around 120 cm) than for the corresponding keto forms (around 190 cm), indicating a more tetragonal or planar geometry. This $g_{//}/A_{//}$ ratio is frequently used to estimate the tetrahedral distortion around a copper ion in a tetragonal environment [48].

**Table 1.** Determined values of spectroscopic parameters (g) and hyperfine constants (A) for oxindolimine–copper(II) complexes [#].

| Complexes at Different pHs | $g_{\perp}$ | $g_{//}$ | $A_{//}$, G | $A_{//}$,* $10^{-4}cm^{-1}$ | $g_{//}/A_{//}$ cm |
|---|---|---|---|---|---|
| [Cu(isaepy)$H_2O$] pH = 3, keto-form | 2.086 | | 112 | 127 | 191 |
| pH = 7 | 2.058 | 2.246 | 173 | 181 | 124 |
| pH = 10, enol-form | 2.059 | 2.252 | 177 | 186 | 121 |
| [Cu(isapn)] pH = 4, keto-keto form | 2.101 | 2.445 | 115 | 131 | 187 |
| pH = 7, keto-enol form | 2.112 | 2.256 | 186 | 196 | 115 |
| pH = 10, enol-enol form | 2.092 | 2.262 | 184 | 194 | 116 |

* $A_{//}(10^{-4}\ cm^{-1}) = g_{//}\beta\ A_{//}(G) = 0.46686 \times 10^{-4}\ g_{//}A_{//}(G)$, where $\beta$ = 1.39969 MHz/G; [#] results adapted and simplified from ref. [49].

$^1$H NMR spectra of the zinc(II) complex, [Zn(isapn)]$ClO_4$ **2** in MeOH-d4, at different pHs in the range of 5 to 9 also indicated the presence of tautomeric equilibria, due to the deprotonation of the NH group at indole ring, as previously verified [42]. Similar results were observed for [Zn(isaepy)$Cl_2$] complex **4**. In this case, three species were detected by ESI-MS data, as shown in Figure S1, corroborated by NMR spectra in Figure S2, in the Supplementary Material.

A signal corresponding to the free ligand *isaepy* occurred at $m/z$ = 252.1 $[M + 1]^+$, [MW = 251.29], attributed to fragment [$C_{15}H_{13}N_3O$]; another fragment at $m/z$ = 350.0 corresponding to the monocation $[Zn(isaepy)(H_2O)]^+ \cdot H_2O$ or [$C_{15}H_{16}N_3O_3{}^{64}Zn$] and 352.0, [MW = 352.04], for [$C_{15}H_{16}N_3O_3{}^{66}Zn$]. Finally, a species Zn:L 1:2 was also verified, since a fragment at $m/z$ = 564.9, [MW = 564.12], was assigned to the monocation $[Zn(isaepy)_2]^+$ [$C_{30}H_{24}N_6O_2{}^{64}Zn$] and $m/z$ = 566.9 [MW = 566.12 for [$C_{30}H_{24}N_6O_2{}^{66}Zn$].

At physiological conditions (pH 7), the keto-enol form seems to be predominant over the corresponding keto-keto or enol-enol forms for both copper(II) and zinc(II) complexes with the ligand *isapn*, as indicated by EPR or NMR data, respectively. For complex $[Cu(isaepy)H_2O]^+$ **3**, the enol form, more planar, with *isaepy* acting as a tridentate ligand, is predominant. For complexes $[Zn(isaepy)]^+$ **4**, and $[Cu(isaepy)_2]^{2+}$ **5**, although isolated as keto forms in solution at pH 7, the enol form seem to be dominant.

All these metal complexes have already shown high stability in solution [48], with relative stability constants of the same order as those of copper(II) or zinc(II) ions inserted in human serum albumin, for which log $K_{[Cu(has)]}$ = 12.0 for copper [50], or log $K_{[hasHSA)]}$ = 7.2 for zinc, have been reported [51].

### 3.2. Evaluation of Trypanocidal Activity

Firstly, complexes **1**, **2**, **4**, and **5** had their toxicity verified against trypomastigote forms of *T. cruzi*, as shown in Figure 3. The corresponding $IC_{50}$ results, corresponding to 50% inhibition of the parasites' viability, are displayed in Table 2.

**Table 2.** $IC_{50}$ values for trypomastigote forms of *T. cruzi* viability ($IC_{50} \pm$ SD *) after 24 or 48 h incubation with the oxindolimine–metal complexes, at 37 °C.

| $IC_{50}$ µmol/L | [Cu(isapn)] $(ClO_4)_2$ | [Cu(isaepy)$_2$] $(ClO_4)_2$ | [Zn(isapn)] $ClO_4$ | [Zn(isaepy)$Cl_2$] |
|---|---|---|---|---|
| 24 h | 15.5 ± 5.5 | 10.7 ± 3.8 | 32.9 ± 14.1 | 80.2 ± 52.6 |
| 48 h | 2.7 ± 1.0 | 3.0 ± 1.0 | 11.3 ± 3.6 | 56.2 ± 23.0 |

* SD = standard deviation.

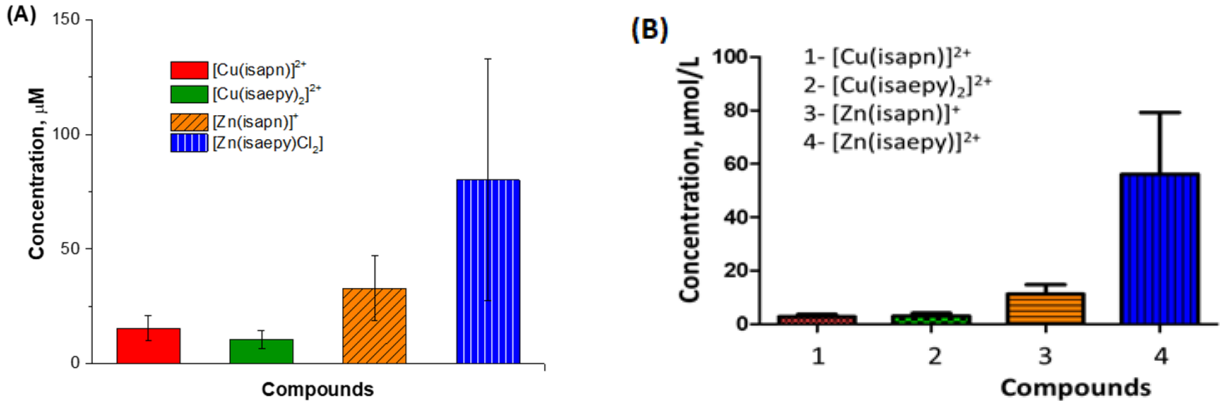


**Figure 3.** $IC_{50}$ values for trypomastigote forms of *T. cruzi* viability, after (**A**) 24 h and (**B**) 48 h incubation with the oxindolimine–metal complexes.

In parallel experiments, the toxicity of such complexes toward macrophages was also determined, as shown in Figure 4 and Table 3.

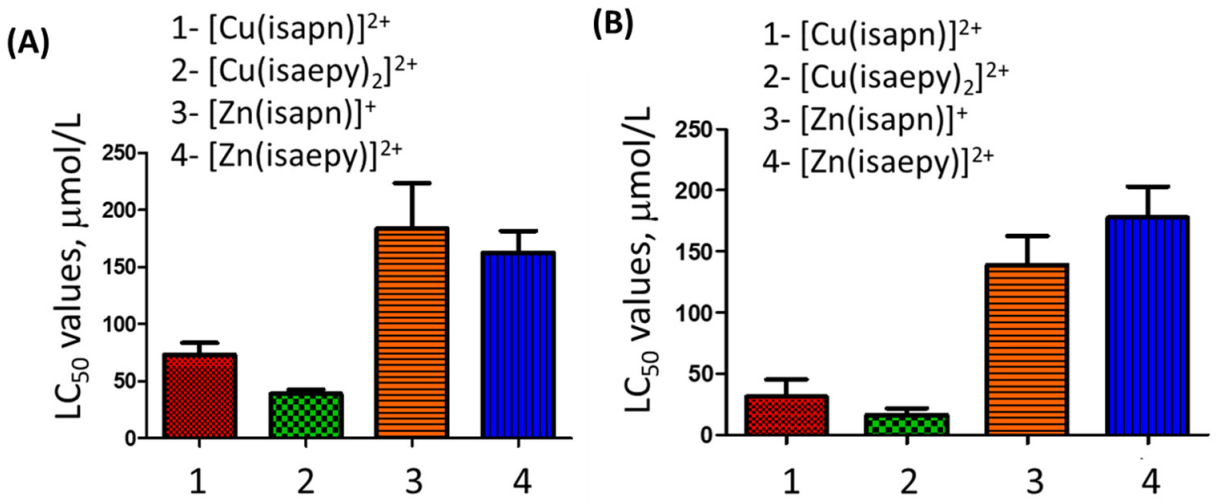


**Figure 4.** Lethal concentrations to 50% of non-infected macrophages ($LC_{50}$) were compared after (**A**) 24 h and (**B**) 48 h incubation with the metal complexes at 37 °C.

**Table 3.** Values of lethal concentration to 50% of non-infected macrophages ($LC_{50} \pm$ SD *) incubated with these complexes for 24 h or 48 h at 37 °C.

| $LC_{50}$ µmol/L | $[Cu(isapn)](ClO_4)_2$ | $[Cu(isaepy)_2](ClO_4)_2$ | $[Zn(isapn)]ClO_4$ | $[Zn(isaepy)Cl_2]$ |
|---|---|---|---|---|
| 24 h | 73.3 ± 10.4 | 39.1 ± 3.5 | 183.8 ± 39.9 | 162.8 ± 18.8 |
| 48 h | 31.3 ± 14.0 | 16.2 ± 5.2 | 138.9 ± 23.8 | 177.8 ± 25.0 |

* SD = standard deviation.

Further, the toxicity of such metal complexes against amastigotes in macrophages of mouse C57BL/6 was also verified. The results are shown in Figure 5 (copper complexes) and Figure 6 (zinc complexes).

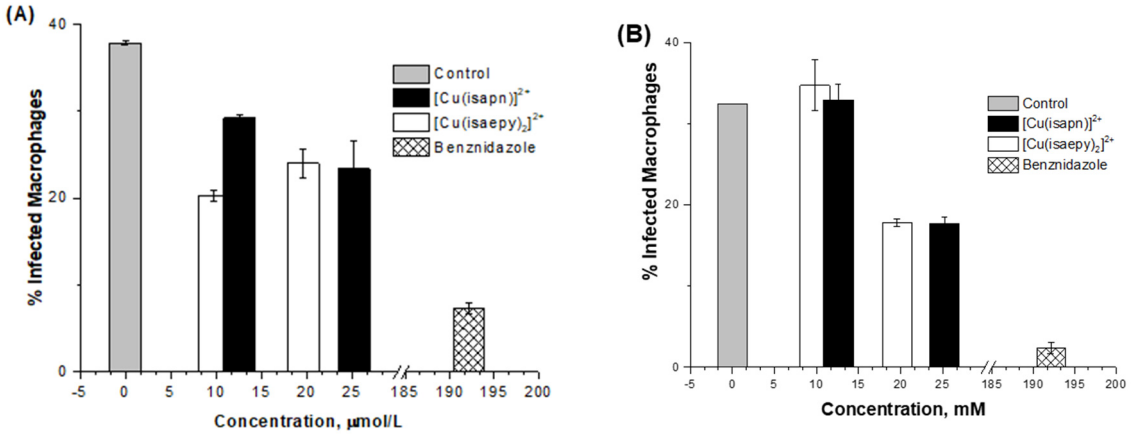


**Figure 5.** Percentage of infected macrophages after (**A**) 24 h and (**B**) 48 h incubation at 37 °C with copper(II) complexes **1** and **5**, in comparison with benznidazole.

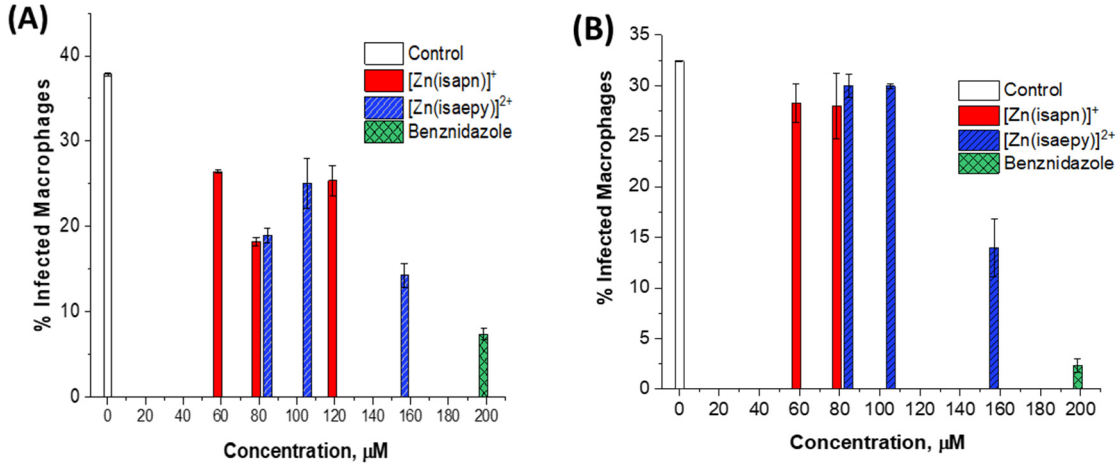


**Figure 6.** Percentage of infected macrophages after (**A**) 24 h and (**B**) 48 h incubation at 37 °C with zinc(II) complexes **2** and **4**, in comparison with benznidazole.

In Table 4, the results and the corresponding selective indexes, macrophages versus trypomastigotes, verified with these complexes are compared to benznidazole.

**Table 4.** $IC_{50}$ values (µM) and corresponding selective indexes (SI) verified against infected macrophages and trypomastigotes, in the presence of the studied metal complexes, after an incubation period of 24 or 48 h, at 37 °C, in comparison to benznidazole.

| | $IC_{50}$ (µM) after 24 h Incubation | | | $IC_{50}$ (µM) after 48 h Incubation | | |
|---|---|---|---|---|---|---|
| **Complex** | **Macrophages** | **Trypomastigotes** | **S.I.** | **Macrophages** | **Trypomastigotes** | **S.I.** |
| $[Cu(isapn)](ClO_4)_2$ **1** | 73.3 ± 10.4 | 15.5 ± 5.5 | 4.8 | 31.3 ± 14.0 | 2.7 ± 1.0 | 11.6 |
| $[Zn(isapn)]ClO_4$ **2** | 183.8 ± 39.9 | 32.9 ± 14.1 | 5.6 | 138.9 ± 23.8 | 11.3 ± 3.6 | 12.4 |
| $[Cu(isaepy)_2](ClO_4)_2$ **5** | 39.1 ± 3.5 | 10.7 ± 3.8 | 3.7 | 16.2 ± 5.2 | 3.0 ± 1.0 | 5.4 |
| $[Zn(isaepy)Cl_2]$ **4** | 162.8 ± 18,8 | 80.2 ± 52.6 | 2.0 | 177.8 ± 25 | 56.2 ± 23 | 3.2 |
| Benznidazole [#] | | 30.3 ± 2.83 | 2.7 | | | |

[#] from ref. [52].

All those metal complexes showed to be efficient toward the trypomastigote forms of parasites, and more active than the free ligands (see Figure 7). Particularly, complexes **1** and **2** were the most active in the series. Copper(II) complexes were more active than the corresponding zinc(II) ones with the same ligand, and the ability of copper compounds to generate ROS is probably responsible for their better performance.

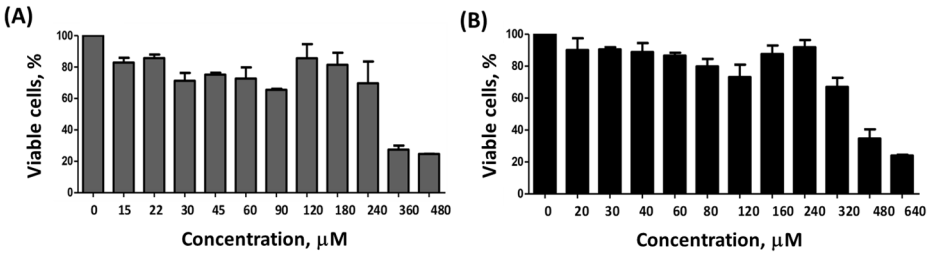


**Figure 7.** Viability of infected macrophages after 48 h incubation with the free ligands (**A**) isapn, and (**B**) isaepy.

Many studies in the literature, however, reported values against the epimastigote forms of the parasite. In Table 5, some already reported data for other metal complexes toward trypomastigotes are displayed, for comparison. In many of them, the incubation times are longer than 24 or 48 h.

**Table 5.** $IC_{50}$ values (μM) and corresponding selective indexes reported in the literature for different metal complexes, at different incubation times (h), against the trypomastigote (or epimastigote [#]) forms of the parasite.

| Complexes | $IC_{50}$ (μM) Trypomastigotes | Selective Index (S.I.) | Incubation Time |
|---|---|---|---|
| $[Cu(dmtp)_4(H_2O)_2]$ $(ClO_4)_2$ dmtp = 5,7-dimethyl-1,2,4-triazolo[1,5-a]pyrimidine | 25.4 ± 2.3 | 16.5 | 72 h [a] |
| $[Zn(dmtp)_2(H_2O)_4]$ $(ClO_4)_2$ | 19.2 ± 1.1 | 3.8 | 72 h [a] |
| $[Cu(4\text{-}MH)(dmb)(ClO_4)_2]\cdot 2H_2O$ 4-MH = 4-methoxybenzhydrazide; dmb = 4-4′-dimethoxy-2-2′-bipyridine | 14.0 | 12.9 | 72 h [b] |
| *trans*-$[Ru(tzdt)(PPh_3)_2(bipy)]PF_6$ tzdtH = 1,3-thiazolidine-2-thione | 0.01 | 34 | 24 h [c] |
| $[Au^{III}(Hdamp)(L1)]NO_3$ (4-$NO_3$) Hdamp = dimethylaminomethylphenyl | 16.9 | 5.1 | 48 h [d] |
| [Pt(HL1)(L1)]Cl [#] L1 = thiosemicarbazone derivative of 1-indanone | (8.7) | (8.8) | 120 h [e] (Epimastigote form) |
| [Pd(HL2)(L2)]Cl [#] L2 = thiosemicarbazone derivative of 1-indanone | (2.3) | (9.5) | 120 h [e] (Epimastigote form) |

[a] ref. [53]; [b] ref. [54]; [c] ref. [55]; [d] ref. [56]; [#] epimastigote form, [e] ref. [25].

These metal complexes were shown to be quite toxic toward both macrophages and trypomastigotes, with good selectivity indexes (S.I.), although after longer times of incubation. Among our oxindolimine complexes, the two derivatives of the *isapn* ligand, complexes **1** and **2**, stand out, because despite presenting less efficiency with higher $IC_{50}$ values, they showed better selectivity indexes after shorter times of incubation.

In further studies, the macrophages viability was also tested in the presence of free ligands isapn and isaepy, as shown in Figure 7.

The cytotoxicity verified in the presence of the free ligands were similar, in the range of 80 to 160 μg/mL, or 320 to 640 μmol/mL for ligand isaepy and 240 to 480 μmol/mL for ligand isapn, respectively, much lower than that of the corresponding zinc(II) or copper(II)

complexes. In Figure 8, the corresponding infection indexes verified after 24 h incubation of macrophages with the free ligands are displayed.

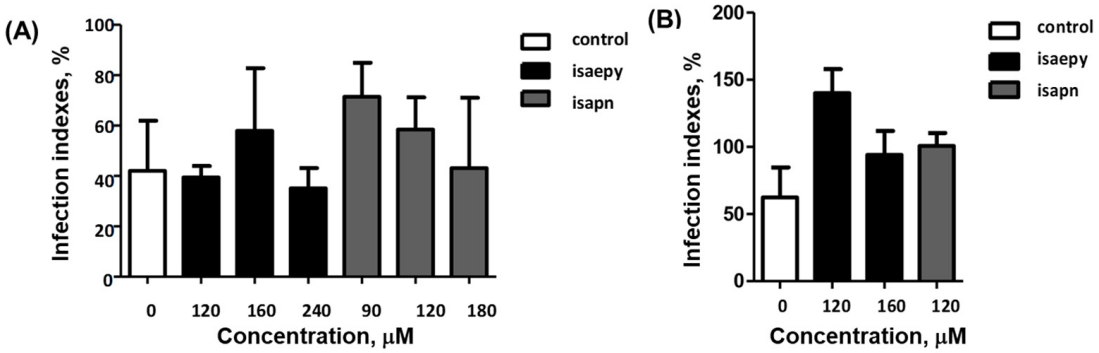


**Figure 8.** Infection indexes after (**A**) 24 h or (**B**) 48 h incubation of the infected macrophages with the free ligands isapn and isaepy at 37 °C.

These results are shown in Table 6.

**Table 6.** Comparison of $IC_{50}$ values at 24 h assays, between two different techniques to measure the trypomastigotes' viability toward the studied copper(II) compounds.

| Complexes | Trypomastigotes (Neubauer Chamber) | Trypomastigotes (MTT) | Correlation (Neubauer Chamber/MTT) |
|---|---|---|---|
| $[Cu(isapn)](ClO_4)_2$ | 15.5 ± 5.5 μM | 6.11 ± 0.44 μM | 2.54 |
| $[Cu(isaepy)_2](ClO_4)_2$ | 10.7 ± 3.8 μM | | |
| $[Cu(isaepy)H_2O]ClO_4$ | | 1.37 ± 0.12 μM | 7.81 |

We can see that the $IC_{50}$ values obtained by the MTT technique were lower than those by reading in the Neubauer chamber, in a ratio that ranged from 2.5× for complex **1** to 7.8× for complex **3** or **5**. This discrepancy can be explained by the difference between the techniques. The optical microscopy with Neubauer chamber counting depends more on the visual acuity of the operator [57], and even if the counting is done meticulously, it is subject to more errors depending on the operator than the MTT method, which carries out the spectrometric reading of the sample operator-independent. Despite this, MTT is not free from errors, requiring extreme care to eliminate influences from the culture medium and possible absorbance of the complexes. The trypomastigotes do not adhere to the well wall, making it impossible to wash them before reading as in the viability test for the macrophage, and requiring the use of controls to reduce the influence of the absorbance of the medium in the measurements.

The oxindolimine–metal complexes also showed toxicity toward the amastigote forms of *T. cruzi*, as shown in Figure 9. The copper complexes were more active (up to 20 μM) than the analogous zinc ones (60 to 120 μM), although less active than benznidazole (160 μM).

Although the generation of ROS can provide a good explanation for the better activity of the copper compounds in comparison to zinc, selected parasite proteins can also be important targets for such metal complexes. Both copper and zinc compounds showed high selective indexes. Kinases have been reported as potential targets for trypanocidal drugs, since there are ~190 protein kinases encoded for *T. cruzi* genomes [58]. Another target ubiquitously studied in the literature is the parasite cruzain protein, essential for the development and survival of the parasite within the host cells [59].

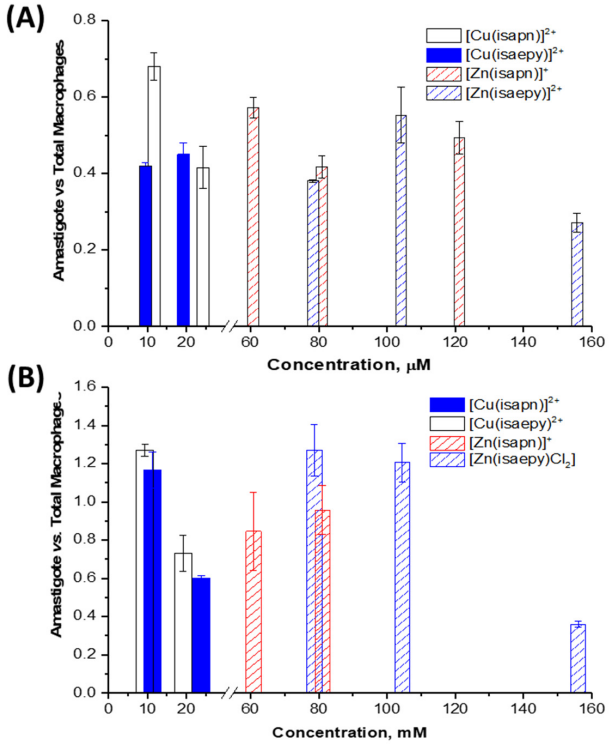


**Figure 9.** Toxicities of oxindolimine–copper(II) and –zinc(II) complexes toward the amastigote forms of *T. cruzi*, after incubation for (**A**) 24 h and (**B**) 48 h at 37 °C.

## 4. Conclusions

All metal complexes reported here showed good activity against the trypomastigote and amastigote forms of *T. cruzi*. For both parasite forms found in humans, the determined $IC_{50}$ values in the presence of the copper(II) complexes are lower than for the analogous zinc(II) compounds, attesting the better activity of copper compounds. However, the estimated selective indexes are better for both copper and zinc compounds with the ligand isapn. In comparison to other metal complexes described in the literature (see Tables 4 and 5), their selective indexes were more favorable after only 24 or 48 h incubation. Further, they were more reactive against protozoa *T. cruzi* than toward *Schistosoma* worms, and their modes of action probably differ in both cases.

Those complexes are very stable thermodynamically, with formation constants of the same order as a copper ion inserted in the N-terminal site of human albumin (log $K_{Cu(HSA)}$ = 12.0) or zinc ion inserted in this protein (log $K_{Zn(HSA)}$ = 7.1), as already reported elsewhere [48]. Further, the active tautomeric forms of these complexes at physiological pH 7.4 are probably the enol ones, corresponding to the deprotonation of the NH group at the indole ring, as demonstrated by the EPR hyperfine parameters, in the case of the copper complexes (Figure 2 and Table 1), or by the NMR spectra, in the case of the zinc complexes [35]. More planar or tetragonal species seem to be more active toward the parasites. The compounds with the isapn ligand were more reactive than the analogous ones with the isaepy ligand, in both copper and zinc complexes.

Additionally, the copper(II) complexes were shown in previous studies to have significant oxidant properties, being able to damage DNA and HSA, in the presence of hydrogen peroxide, with the formation of hydroxyl radicals. Therefore, an explanation for its antiparasitic activity could be the induction of oxidative stress, damaging membranes, and vital molecules in the parasite, through ROS formation [38]. However, previous results also revealed that interactions of such oxindolimine complexes with specific proteins are in the same way determinant of their biological activity. The same order of reactivity has been demonstrated for such metal complexes regarding the inhibition of topoisomerase IB protein [41]. Also in these studies, the [Cu(isapn)] complex **1** was a more active inhibitor than

[Cu(isaepy)] complex **3** or **5**, and the copper species with each of these ligands were more efficient than the corresponding zinc species. Nevertheless, further studies are necessary to elucidate the probable modes of action of these oxindolimine–metal complexes, and to provide other new and more efficient compounds as trypanocydal agents, based on our results. Further mechanistic studies are in progress in our laboratory to identify probable parasite targets.

## 5. Patents

The University of São Paulo has filed patent applications (AUCANI—USP Innovation Agency) related to the antiparasitic activity of the oxindolimine–metal complexes under study in our laboratory (INPI, **BR 10 2013 026558 6**). This patent has not been conceded yet, it is still under evaluation. A related Brazilian patent, regarding anticancer activity of this class of metal complexes, was conceded on 24 March 2020 (**BR 2006 00985-A**).

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/inorganics11110420/s1, Figure S1: Mass spectrogram of complex [Zn(isaepy)$Cl_2$] **4** in methanol:water (9:1) solution; Figure S2. $^1$H NMR spectra of (A) isaepy free ligand, and (B) complex **4** [Zn(isaepy)$Cl_2$] in $D_2O$.

**Author Contributions:** A detailed description of the diverse contributions of the co-authors to the published work. G.L.S.: investigation, formal analysis, validation, and writing—original draft preparation. M.C.P.: methodology, investigation, formal analysis, validation, and writing—original draft preparation. G.A.R.: investigation, methodology, and formal analysis. R.A.A.D.C.: methodology, investigation, formal analysis, and visualization. L.Q.V.: conceptualization, investigation, writing—review and editing, and resources. M.J.M.A.: conceptualization, investigation, resources, and writing—review and editing. A.M.D.C.F.: conceptualization, methodology, supervision, writing—review and editing, and funding acquisition. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** Data are available on request.

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**Conflicts of Interest:** The authors declare no conflict of interest.

## Abbreviations

| | |
|---|---|
| Cruzain | a recombinant form of protein cruzipain, EC 3.4.22.51 |
| DPPH | α,α′-diphenyl-β-picrylhydrazyl |
| EPR | electron paramagnetic resonance |
| HGPRTs | hypoxanthine−guanine phosphoribosyl-transferases |
| isaepy | (*E*)-3-((2-(pyridin-2-yl)ethyl)imino)indolin-2-one; oxindolimine ligand obtained from isatin and 2-(2-aminoethyl)pyridine |
| isapn | (3*E*,3′*E*)-3,3′-(propane-1,3-diylbis(azaneylylidene)bis(indolin-2-one); oxindolimine ligand obtained from isatin and 1,3-diaminopropane |
| U2AF35 | host protein that binds to RNA at the polypyrimidine tract |

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