

Synthesis, characterization and evaluation of antileishmanial activity of copper(II) with fluorinated α -hydroxycarboxylate ligands

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Abstract In this study, Cu(II) complexes with fluorinated ligands were produced aiming at the development of new, less toxic antileishmanial metal-lodrugs. Complexes of the general formula CuL_2 (L = lactate, trifluorolactate, 2-hydroxyisobutyrate, trifluoro-2-hydroxyisobutyrate) were synthesized in methanolic medium, purified by crystallization and characterized by elemental analysis and electronic and infrared spectroscopies. In vitro experiments with *Leishmania amazonensis* promastigotes showed that the trifluorolactate derivative more active than its non-fluorinated counterpart. Our results indicate that fluorinated chelators may be interesting to increase metal toxicity and/or open new paths for metallodrugs chemotherapy against leishmaniasis.

Keywords Leishmaniasis · Neglected disease · Copper · Fluorine

Introduction

Leishmaniasis is an infection caused by protozoa of the *Leishmania* genus, which infect macrophages of vertebrate hosts resulting in effects such as skin ulceration, mucous membrane destruction and general infection of viscera. These symptoms define, respectively, the cutaneous, muco-cutaneous or visceral forms of the disease. The leishmaniasis cycle starts when phlebotomine insects (sandfly, genus *Lutzomyia* or *Phlebotomus*) inoculate the parasite in the promastigote phase into the vertebrate host, leading to invasion and/or quick phagocytosis by neutrophils and macrophages. In the macrophages, the surviving parasites multiply as amastigotes which will infect other cells throughout the organism. Host inflammatory and immune responses are ineffective to check the progression of the disease, leading to the progressive development of clinical symptoms in a period of weeks to months. It is estimated that 1–1.5 million new cases of cutaneous leishmaniasis and 0.5 million new cases of visceral leishmaniasis develop annually, with a prevalence of 12 million patients in the world, the vast majority in poor or underdeveloped nations (Murray 2005).

The therapeutic arsenal to treat leishmaniasis is relatively limited, based mostly on antimonial compounds and a few other drugs. Examples of the latter include amphotericin B, introduced in the mid-1980s, pentamidine and, more recently, miltefosine (Croft et al. 2005; Murray 2005). Several other molecules,

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such as nerolidol (Arruda 2005), modified chalcones (Boeck 2006) and several 2,4-diamminoquinazolines (Khabnadideh 2005) have been shown to be active in vitro and/or in experimental models of the disease, but none of these have been approved for clinical trials.

Antimony compounds have long been used to treat leishmaniasis, the first of which was emetic tartar (antimony(III) tartrate). However, the high toxicity of antimony (III) results in serious side effects (gastro-intestinal intolerance, cardiotoxicity, nephrotoxicity) which have rendered emetic tartar useless for practical purposes (Rath et al. 2003). Since then, trivalent antimonials have been substituted by less toxic pentavalent derivatives such as the sugar complexes sodium stibogluconate (Pentostan®) and meglumine antimoniate (Glucantime®) (Marsden 1985; Berman 1988).

The mechanism of action of pentavalent antimonial compounds is not fully understood. They are probably reduced in vivo by parasite thiols (tripanothione) to the trivalent, more toxic forms, suggesting that the Sb(V) complex acts as a pro-drug. The higher toxicity of the reduced form is probably related to its “softer” character and therefore its preference to bind to sulfur-containing biological substrates responsible for the redox balance of the parasite (e.g., tripanothione). Nucleoside binding and inhibition of DNA repair, followed by induction of apoptosis, is another biochemical route prone to be disturbed by Sb(III) species (Yan et al. 2005). However, Sb compounds are also harmful to the host, leading to serious side effects. Furthermore, resistance to pentavalent antimonials has emerged and it is now widespread in some areas, particularly on the Indian subcontinent (Croft et al. 2006). Therefore, the search for other active metal complexes persists (Fricker et al. 2008).

Copper as a metal center of choice appears as an interesting alternative, since its complexes are usually less toxic than their Sb congeners. Also, if the “activation by reduction” mechanism is in fact important, copper has reduced forms (based on Cu^+) which are more toxic than the oxidized ones (Lim et al. 2006).

Fluorination confers interesting properties to organic compounds. Fluorine is a small, highly electronegative, atom, with a smaller volume than groups such as CH_3 , NH_2 and OH. The C–F bond is highly polar but only slightly polarizable, accounting

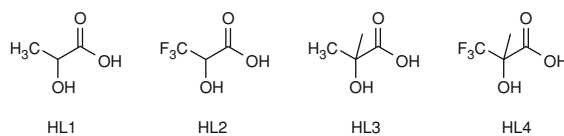
in some instances for the low aqueous solvation energies and high lipophilicities of some fluorinated molecules in comparison with their non-fluorinated analogs (Kim 1998; Biffinger et al. 2004; Gerebtzoff 2004; Shimizu and Hiyama 2005). Fluorine is already widely present in medicine: currently, there are ca. 130 drugs containing at least one F atom in their composition (Bohm 2004). Other benefits of organic fluorination may include increased metabolic stability, increased affinity for a substrate, and altered nucleophilicity. Substitution of an H for an F atom in a drug molecule often improves its bioavailability (Gerebtzoff 2004; Shimizu and Hiyama 2005).

In this study we prepared new fluorinated α -hydroxycarboxylate derivatives of Cu(II) in order to compare the effect of this functionalization on the anti-proliferative activity of the complexes towards *Leishmania*. The α -hydroxycarboxylate motif was chosen as a simplified version of the coordination mode of carbohydrates in antimonial antileishmanial drugs. We observed that bis(trifluorolactato)copper(II) was the most active against promastigotes of *Leishmania amazonensis* promastigotes, suggesting that fluorination may account for increased toxicity in some instances.

Materials and methods

Chemicals: $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (Cromoline, Brazil); methanol, D,L-lactic acid, 2-hydroxypropionic acid (Sigma); trifluorolactic acid, 2-(trifluoromethyl)-2-hydroxypropionic acid (Matrix Scientific) were used without further purification. The structures of the ligands are depicted in Structure 1.

Synthesis: The complexes were formed by the treatment of 0.75 M CuSO_4 aqueous solutions with equal volumes of aqueous solutions of the ligands (L) in order to attain 1:2 Cu:L final molar ratios. The mixtures were stirred at room temperature;



Structure 1 Structures of the ligands. HL1 = lactic acid; HL2 = trifluorolactic acid; HL3 = 2-hydroxypropionic acid; HL4 = 2-(trifluoromethyl)-2-hydroxypropionic acid

Table 1 Elemental analysis results for the copper complexes

	%C		%H	
	Calc.	Exp.	Calc.	Exp.
Cu(L1) ₂ (H ₂ O) ₂	25.95	25.61	5.08	5.13
Cu(L2) ₂ (H ₂ O) ₅	16.47	16.12	2.76	2.44
Cu(L3) ₂ (H ₂ O) ₂	31.42	31.72	5.93	5.41
Cu(L4) ₂ (H ₂ O) ₂	23.23	23.30	2.92	2.72

immediately after mixing the reagents the development of different hues of blue was observed, depending on L. The pH was adjusted to 7 with NaOH(aq) in order to ensure total deprotonation of the carboxylic moiety of L. Finally, the solutions were treated with 4×100 ml methanol and a white precipitate was observed (probably NaOH in excess or Na₂SO₄) and discarded. The blue methanolic solutions were concentrated to 20 ml and kept in the refrigerator for 2 days. The crystals were filtered, washed once with cold methanol and oven dried. The elemental analysis results follow in Table 1.

Instrumentation: Infrared spectra were obtained in KBr pellets in a Bomem MB100 instrument. Electronic spectra were acquired in a Tecan Sapphire instrument at room temperature.

Partition studies: Distribution of the copper complexes through multilayer lecithin vesicles (MLV) was studied as a means to assess lipophilicity in a biologically relevant model (Engelmann et al. 2007). In 1.5 ml plastic vials, 100 μ l of Cu(II) complexes *ca* 30 mM were added to 100 μ l of buffered MLV suspension prepared as previously described (Engelmann et al. 2007). The vials were vortexed by 5 min at room temperature, centrifuged and the absorbance of the supernatants was determined at the λ_{max} values of each metal complex (see Table 2). Partition coefficients were determined by the ratio of the absorbance values after and prior the treatment with MLV.

Table 2 Maximum wavelengths (λ_{max}) in different solvents

Compound	H ₂ O	dmsO
Cu(L1) ₂	762	734
Cu(L2) ₂	732	700
Cu(L3) ₂	770	748
Cu(L4) ₂	760	742

Biological studies: Promastigotes of *L. amazonensis* were grown in Medium 199 supplemented with 10% fetal calf serum (FCS) at 25°C as described previously (Arruda 2005; Souza et al. 2006). Inhibitory concentrations of the copper compounds were determined by seeding 1.3×10^6 promastigotes/ml in the presence of increasing concentrations of the copper complexes (1–1,000 μ M), in 96-well culture plates (Corning Life Sciences, Corning, NY) for 48 and 72 h. Quantification of viable cells was assessed by cell counting in Neubauer chambers after 48 and 72 h of incubation with the test solutions. Assays were performed in triplicate, and results are expressed as the mean percent reduction of parasite numbers compared to untreated control wells calculated for at least two independent experiments. The 50% inhibitory concentrations (IC₅₀) were determined from sigmoidal regression of the concentration-response curves using Scientific Graphing and Analysis Software ORIGIN 7.5.

Results and discussion

Fluorination of the ligand induces hypsochromic shifts in the visible spectra of the complexes of *ca.* 10 nm (Cu(L3)₂ $\rightarrow$ Cu(L4)₂) and 30 nm (Cu(L1)₂ $\rightarrow$ Cu(L2)₂), Fig. 1 and Table 2. This effect is observed both in water and in dimethyl sulfoxide (dmsO). These figures correlate with the increased π -acidity of the ligands provided by the CF₃ groups, rendering L2 and L4 with an overall better π -acceptor character than their non-fluorinated analogs and thus increasing the Δ_O values in their complexes (Shriver and Atkins 1999). Positive inductive effects of the methyl groups in L3 and L4 may compensate, to some extent, the electron-removal effect of the CF₃ group, which might explain the smaller shift observed for the Cu(L3)₂ $\rightarrow$ Cu(L4)₂ substitution. For any of the complexes, exchanging water for dmsO as the solvent leads to a hypsochromic shift of *ca.* 30 nm (Cu(L1)₂, Cu(L2)₂) or 20 nm (Cu(L3)₂, Cu(L4)₂). This effect is correlated with the better π -acceptor character of dmsO as compared to water. Also, molar absorptivity values of *ca.* 130 M^{−1} cm^{−1} are consistent with *d–d* transitions (Shriver and Atkins 1999).

Inspection of infrared spectra (Fig. 2) revealed characteristic frequencies of the organic moiety of the complexes (Table 3) which allowed us to confirm their

Fig. 1 Visible electronic spectra of *ca* 10 mM aqueous solutions of the complexes

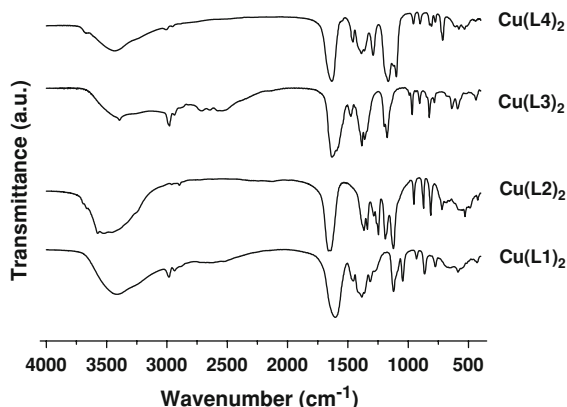
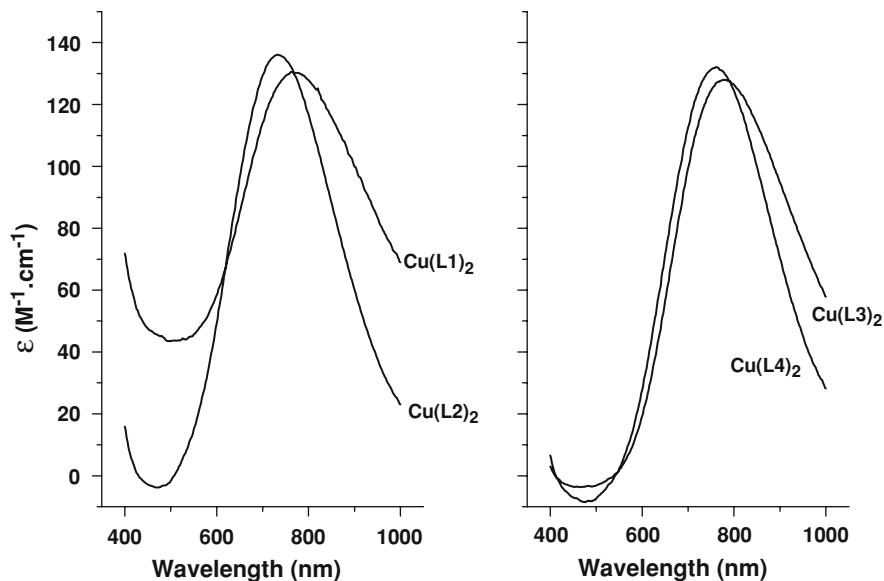


Fig. 2 Infrared spectra in KBr pellets of the copper complexes

coordination modes. Both $\nu_{as}(\text{COO})$ and $\nu_s(\text{COO})$ vibrations are coherent with deprotonated carboxylates (theoretical ranges of 1,550–1,650 and 1,400 cm^{-1} , respectively (Goulden 1960; Silverstein et al. 2005)), with $\Delta(\text{COO})$ values of 200–300 cm^{-1} indicating monodentate carboxylate binding to the metal center (Deacon and Philips 1980). Frequencies

at *ca.* 3,500 cm^{-1} are attributable to $\nu(\text{OH})$ at the α -carbon (Silverstein et al. 2005), which remained comparatively unchanged in comparison to the respective parent α -hydroxycarboxylic acids (data not shown), indicating that this oxygen remains protonated during coordination. Frequencies of around 1,000–1,260 cm^{-1} may be attributed to $\nu(\text{CO})$ of the α carbon—OH stretch. Bands at 1,100–1,350 and 700–750 cm^{-1} appear only in the fluorinated complexes and are indicative of $\nu(\text{CF})$ stretching frequencies (Silverstein et al. 2005).

α -hydroxycarboxylic acids behave as bidentate ligands toward Cu(II) (Dillon and Rossotti 1966), producing neutral 1:2 complexes with lactate (Bobtelsky and Bar-Gadda 1953; Mohanty and Patnaik 1984) and other compounds (Goulden 1960; Forrest et al. 1966; Prout et al. 1968; Buravchuk et al. 1974). A pH adjustment to neutral is required to keep the carboxylic moiety of the ligands deprotonated, however, in all instances the α -OH groups coordinate to the metal without deprotonation. The Jahn-Teller effect stabilizes Cu(II) ions in a tetragonally distorted

Table 3 Selected infrared frequencies (cm^{-1}) for the copper complexes in KBr pellets

Compound	$\nu_{as}(\text{COO})$	$\nu_s(\text{COO})$	$\Delta(\text{COO})$	$\nu(\alpha\text{OH})$	$\nu(\text{CO})$	$\nu(\text{CF})$
Cu(L1) ₂	1,609	1,391	218	3,447	1,124	
Cu(L2) ₂	1,666	1,371	295	3,570	1,126	1,248, 723
Cu(L3) ₂	1,647	1,387	260	3,402	1,176	
Cu(L4) ₂	1,643	1,393	250	3,472	1,173	1,296, 716

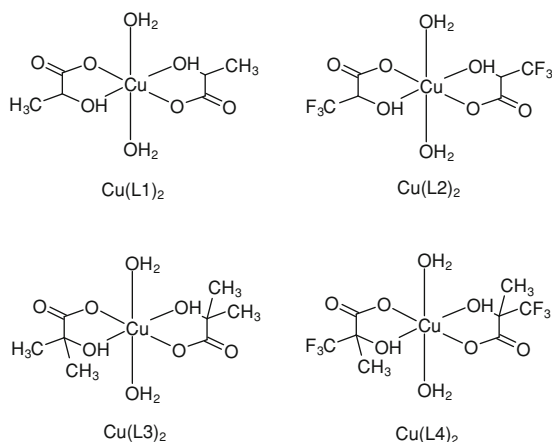


Fig. 3 Proposed structures for the Cu(II) complexes in this study

octahedral environment, with two solvent molecules in the axial positions and two bidentate ligands *trans* to each other in the plane. α -hydroxycarboxylates coordinate through one oxygen of the carboxylate group and through the hydroxyl oxygen at the alpha carbon, which remains protonated (Bolard 1965; Carballo et al. 2001). Our infrared data indicate that our complexes fit this structural motif, and their proposed structures are shown in Fig. 3.

All the complexes are fairly soluble in water, with solubilities of *ca.* 1 g ml⁻¹ (Cu(L1)₂, Cu(L3)₂, Cu(L4)₂) or 0.2 g ml⁻¹ (Cu(L2)₂) at 25°C. Despite considerable water solubility, copper trifluorolactate (L2) was the most hydrophobic compound when challenged to multilayer lecithin vesicles (MLV; Table 4), forming metal complexes of amphiphilic character as observed in other metal fluorocarboxylates (Esposito et al. 1999).

Table 4 IC₅₀ values (μ M $\pm$ SD; *n* = 3) of the copper complexes after 48 and 72 h of treatment on promastigotes of *L. amazonensis* and partition coefficients (*P*) in multilayer lecithin vesicles

Compound	48 h	72 h	<i>P</i>
CuSO ₄	218.4 $\pm$ 40.0	106.5 $\pm$ 11.1	–
Cu(L1) ₂	242.9 $\pm$ 32.1	292.7 $\pm$ 11.4	0.055
Cu(L2) ₂	257.7 $\pm$ 42.5	166.7 $\pm$ 18.3	0.152
Cu(L3) ₂	339.6 $\pm$ 41.0	187.4 $\pm$ 21.5	0.040
Cu(L4) ₂	412.4 $\pm$ 94.7	272.9 $\pm$ 21.3	0.082

The biological activities of the Cu(II) complexes and CuSO₄ were tested in promastigotes of *L. amazonensis* (Fig. 4) and cytotoxicity was also determined against a mammalian cell line (HeLa). The parent α -hydroxycarboxylic acids were also tested but displayed virtually no activity (data not shown).

Dose-response profiles for the parasite obtained at different time intervals indicated maximum activity against the parasites at 72 h (Table 4). There was no statistically significant differences (*P* > 0.05) in the IC₅₀ values of the copper complexes and CuSO₄, indicating that the complexes keep the same biocidal activity of the “free” ion. Along with the fact that the α -hydroxycarboxylic acids displayed no activity, it seems clear that the chelator role in these complexes is thus to ferry the toxic metal center in a stable way to its target. Chelation allows for better targeting and protection against possible side reactions, making it possible, in some instances, for the complex to be more active than either the metal or ligands alone, as demonstrated for a mixture of Cu(II) and lactic acid against *Escherichia coli* or a number of different *Salmonella* strains in different growth media (Ibrahim et al. 2008). This is very important for systemic metallodrug chemotherapy, which can not rely on the simple administration of metal salts due to the possibility of a multitude of side reactions. Specifically, Cu(II) has a very high affinity binding site in the abundant plasma protein albumin (Harford and Sarkar 1997), therefore copper chelation is important to protect the metal ion and decrease administered doses. Our results indicate that we were successful in

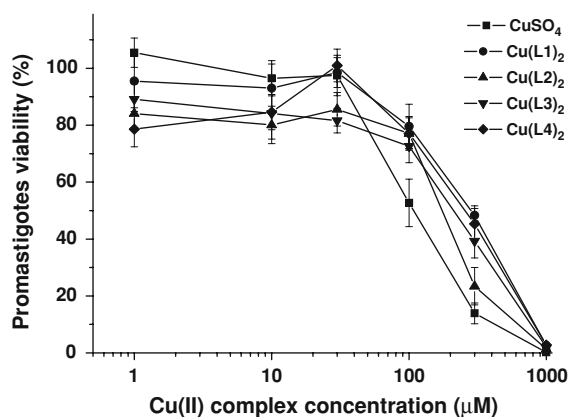


Fig. 4 Dose-response curves after 72 h for promastigotes of *L. amazonensis* incubated with the copper compounds

producing biologically active copper complexes which retain the biological toxic activity of the copper ion at the same time that may allow for enhanced stability towards undesired side reactions involving the metal center and non-targets.

Despite the similarity of the antileishmanial activities (Table 4; Fig. 4), hydrophobic copper trifluorolactate was more toxic than its non-fluorinated analog copper lactate, probably due to enhanced parasite membrane permeation of the former compound. CF_3 and CH_3 possess opposite inductive effects, and the insertion of both groups at the same time (as in $\text{Cu}(\text{L4})_2$) seems to reduce the therapeutic value of the final complex. All the copper species induced a maximum of *ca.* 65% toxicity toward mammalian (HeLa) cells at their highest concentrations, allowing us to estimate their IC_{50} values at higher than 300 μM , i.e., two to three times higher than the values for the promastigotes (data not shown). Further studies in infected animals are needed in order to assess whether this difference is therapeutically useful.

Other metallodrugs have been developed for antileishmanial therapy. Platinum bound to sterol hydrazones in concentrations of *ca.* 10 μM decreased the proliferation of *L. mexicana* promastigotes by around 70% (Visbal et al. 2008). Parasitic cysteine proteases have been successfully inhibited by a plethora of complexes of Au(III), Pd(II) and Re(V) compounds, with IC_{50} values ranging from 0.009–1.4 μM (Fricker et al. 2008). DNA intercalators based on Cu(II) (Navarro et al. 2003a, b) or Au(I) (Navarro et al. 2007) are interesting antileishmanial drugs; $[\text{Au}(\text{dppz})_2]\text{Cl}_3$ showed a marked antiproliferative activity against *L. mexicana*, with 17 nM LD26 after 48 h. Here, we showed that fluorination of Cu(II) complexes may increase their activity (provided that inductive effects are taken into account), and probably increase the activity of other complexes as well.

Conclusions

We showed in this work that Cu(II) complexes with α -hydrocarboxylates retain the toxic activity of copper ions towards *L. amazonensis*. The strategy of ligand fluorination allowed us to develop a lipophilic complex (bis(trifluorolactato)copper(II)) whose antileishmanial activity against *L. amazonensis* was higher than

the activity of its non-fluorinated analog, indicating that ligand fluorination may prove to be an alternative to the rational design of new, more active antileishmanial metallodrugs.

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