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ORIGINAL PAPER

Increasing the activity of copper(II) complexes against *Leishmania* through lipophilicity and pro-oxidant ability

Andressa dos Santos Portas · Danilo Ciccone Miguel · Jenicer K. U. Yokoyama-Yasunaka · Silvia Reni Bortolin Uliana · Breno Pannia Espósito

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Abstract Copper complexes with fluorinated β-diketones were synthesized and characterized in terms of lipophilicity and peroxide-assisted oxidation of dihydrorhodamine as an indicator of redox activity. The biological activity of the complexes was tested against promastigotes of *Leishmania amazonensis*. Inhibition of trypanosomatid-specific trypanothione reductase was also tested. It was found that the highly lipophilic and redox-active bis(trifluoroacetylacetonate) derivative had increased toxicity towards promastigotes. These results indicate that it is possible to modulate the activity of metallodrugs based on redox-active metals through the appropriate choice of lipophilic chelators in order to design new antileishmanials. Further work will be necessary to improve selectivity of these compounds against the parasite.

Keywords Copper · Leishmaniasis · Lipophilicity · Dihydrorhodamine · Fluorescence

Introduction

Leishmaniasis is one of the most burdening tropical diseases, threatening about 350 million people around the world, especially in poor and underdeveloped countries. Despite it being long known to human populations, there are still no vaccines to prevent this disease and the available drugs are either too toxic (such as pentavalent antimonials) or expensive (such as the antifungal preparation AmbiSome) to be efficient in the most afflicted regions [1], and thus the ongoing search for new drugs within both industry and academy is well justified.

There are several interesting concepts related to how metal transport and metabolism in *Leishmania* may be used advantageously to design new therapies. These include metal complexes which function as inhibitors of parasite-specific enzymes such as cysteine proteases [2], aquaporin, aquaglyceroporin [3], peptidase [4], and fumarate reductase [5]. Antimony compounds were found to inhibit parasite-specific trypanothione reductase (TR) [6–8], an enzyme found both in *Trypanosoma* and *Leishmania* spp. which is responsible for the maintenance of the redox balance of trypanosomatids. TR is considered a chemotherapeutic target common to all trypanosomal and leishmanial diseases [9–11]. Regulation of immune response by zinc supplementation has been found to be beneficial [12]. Also, modulating the iron metabolism either through inhibiting the *Leishmania*-specific ferrous transporter LIT1 [13] or through chelation therapy targeted to the ribonucleotide reductase of the parasite [14] seems to be a valid therapeutic approach.

Several new metallodrugs have been proposed for antileishmanial therapy, such as rhenium [15], ferrocenyl [16], copper [17], and gold [18, 19] derivatives, to name a few. Recently, attention has been focused on the possibility of using gold or (magnetic) iron oxide nanoparticles as carriers for drugs to treat *Leishmania* in vitro and/or clinically [20]. Also, metal nitrosyls such as ruthenium nitrosyls [21] or nitroprusside [22] may deliver toxic NO site-specifically to desired targets, and in the case of

A. d. S. Portas · B. P. Espósito (✉)
Instituto de Química,
Universidade de São Paulo,
Av Lineu Prestes 748,
05508-000 São Paulo, SP, Brazil
e-mail: breno@iq.usp.br

D. C. Miguel · J. K. U. Yokoyama-Yasunaka · S. R. B. Uliana
Instituto de Ciências Biomédicas,
Universidade de São Paulo,
Av Lineu Prestes 1374,
05508-000 São Paulo, SP, Brazil

nitroprusside the antiparasitic activity seems to involve major redox disruption.

Copper complexes based on fluorinated α-hydroxycarboxylates have been previously investigated for use in antileishmanial therapy [23], and the results indicated that lipophilic complexes may be best suited to access the parasite. Intercalation with DNA is also a useful strategy in the design of more active Cu(II) complexes [17]. The "S_2 complex," a combination of copper chloride, ascorbate, and nicotinamide, has been found to have a direct antileishmanial effect [24]. Copper ions were found to increase the antileishmanial effect of nitrobenzenesulfonamides (resulting in nuclease activity [25]) and disulfiram [26].

Copper species have long been known to be powerful catalysts of oxidative damage both in vitro and in vivo when challenged by endogenous substrates such as ascorbate, thiols, peroxynitrite, and peroxides [27]. The true nature of the oxidant generated by the reaction of Cu(II) with H_2O_2 remains elusive, possibly involving either the formation of singlet oxygen or a highly reactive Cu(III) intermediate [28, 29]. Disruption of the redox balance of selected targets has been proposed as a valid therapeutic approach to fight disease [30–33]. Therefore, in this study we determined whether integration of adequate lipophilic and redox-active properties in the Cu(II) complexes may be useful to kill *Leishmania* promastigotes using reactive species generated by the reaction of copper complexes with endogenous peroxide. Specifically, we established that the enhancement of both lipophilicity and the H_2O_2-mediated oxidation rate results in more efficient Cu(II) antileishmanial compounds. Also, lipophilic complexes are better inhibitors of trypanosomatid-specific TR.

Materials and methods

$CuSO_4 \cdot 5H_2O$, nitrilotriacetic acid (nta), acetylacetone (acac), trypanothione disulfide (TS_2) trifluoroacetate salt, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), NADPH, dimethyl sulfoxide, and *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid (HEPES) were purchased from Sigma. Trifluoroacetylacetone (tfacac) and hexafluoroacetylacetone (hfacac) were from SynQuest. Clomipramine hydrochloride was from Tocris. TR from *Trypanosoma cruzi* (approximately 20 μM in 70% ammonium sulfate) was donated by Alan Fairlamb (Dundee, UK). Dihydrorhodamine hydrochloride (DHR) was obtained from Biotium and *n*-octanol was obtained from Cromoline (Brazil). Working solutions of the bischelates [34, 35] $Cu(nta)_2$, $Cu(acac)_2$, $Cu(tfacac)_2$, and $Cu(hfacac)_2$ were prepared by mixing appropriate volumes of stock solutions of $CuSO_4$ (90 mM) and ligand (100 mM, pH adjusted to 7 with concentrated NaOH) to attain a final copper-to-ligand ratio of 1:2 (Scheme 1).

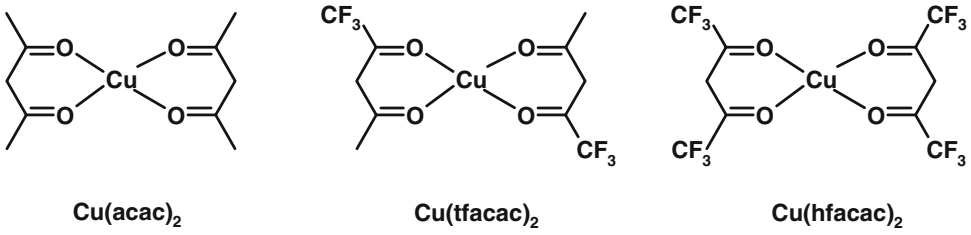
Octanol–water partition coefficients (*P*) were determined by the shake-flask method [36] starting with 50 mM (in Cu) aqueous solutions of the complexes. Copper concentrations of the aqueous supernatants were determined spectrophotometrically in a Tecan Safire reader by monitoring the characteristic absorption maxima of the complexes at 730–780 nm.

The pro-oxidant activity of the copper complexes in the presence of H_2O_2 was assessed by a fluorescence method described elsewhere [37]. Aliquots (10 μL) of 5 μM copper species were transferred to 96-well, transparent, flat-bottom microplates and treated with 10 μL of H_2O_2 (0–200 μM) and 180 μL of 50 μM DHR in HEPES-buffered saline (20 mM HEPES, 150 mM NaCl, pH 7.4, treated with Chelex®-100, 1 g/100 mL). Assays were performed in duplicate. Fluorescence was measured in a BMG FLUOstar Optima plate reader for 40 min at 37 °C (excitation wavelength 485 nm, emission wavelength 520 nm). Peroxide solution in the absence of metal complexes was used as the blank.

Studies on TR inhibition were performed using the original method of Hamilton et al. [38] with slight modifications. TS_2 (0.8 μM), TR (0.75 μM), HEPES (40 mM), and NADPH (1.2 mM) were preincubated in 96-well microplates (250-μL final volume) for 30 min at 30 °C in the presence of inhibitors (10 μM clomipramine or 50 μM copper complexes). No EDTA was added and thus copper redistribution between chelators was precluded. After incubation the samples were treated with 25 μM DTNB in dimethyl sulfoxide and the absorbance at 405 nm was recorded for 5 min in a BMG FLUOStar Optima plate reader. Slopes were obtained for the kinetic curves. All tests were conducted in triplicate.

Biological assays were conducted as described previously [23]. Briefly, *Leishmania amazonensis* (MHOM/BR/1973/M2269) promastigotes, grown in Medium 199 (Sigma-Aldrich) with 10% fetal calf serum (Invitrogen) at

Scheme 1 Proposed structures for the Cu(II) diketonates



25 °C, were incubated with increasing concentrations (1–1,000 μM) of copper compounds for 24, 48, and 72 h in 96-well plates. The viability of triplicate test samples was assessed for each dose tested by cell counting in Neubauer chambers. Each experiment was repeated twice. The results presented herein are expressed as the 50% effective concentration (EC_{50}) calculated from sigmoidal regression of the dose–response curves using Origin 7.5.

Cytotoxicity was evaluated by cultivating 1×10^6 Vero cells (monkey kidney epithelial cells) in RPMI medium (Invitrogen) supplemented with 10% fetal calf serum at 37 °C in 24-well plates for 24, 48, or 72 h in the presence of increasing concentrations of each compound. Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described in [39]. The EC_{50} values for each complex were determined as described above.

Statistical analysis was performed using Prism 4.0 (GraphPad Software, San Diego, CA, USA). The significance of the differences between the groups was examined by analysis of variance followed by the Bonferroni test. Pearson's correlation was performed for the examination of the interrelations between groups. Significance was considered when $P < 0.05$.

Results and discussion

Copper β-diketonates are well known for their use in technological applications, which include the metal vapor deposition of metals and oxides, and as starting materials for supramolecular chemistry [40], although biologically active derivatives are produced [41–44]. The pro-oxidant activity of $Cu(nta)_2$ renders it a potent carcinogen and DNA splicer [45, 46], although it can be beneficial as a copper supplement in cases of copper metabolism dysfunction [47]. In this article, the antileishmanial activities of the copper(II) β-diketonates $Cu(acac)_2$, $Cu(tfacac)_2$, and $Cu(hfacac)_2$ and of $Cu(nta)_2$ are reported for the first time.

Previous studies by our group with fluorinated copper complexes [23] indicated that fluorination leads to an increase in lipophilicity which has a positive effect in the design of more efficient antileishmanial metallodrugs. In this study we pursued the obtainment of both lipophilic and redox-active copper compounds that can activate endogenous peroxide and lead to redox disruption within the parasite. Because they are based on an essential element, it can be assumed that copper compounds have less severe adverse effects than the standard antimonials used in leishmaniasis therapy.

The lipophilicity values for the different complexes are reported in Table 1. As anticipated, substitution of a CH_3 group by a CF_3 group resulted in increased lipophilicity of $Cu(tfacac)_2$ as compared with $Cu(acac)_2$ [23, 48]. Indeed, the trifluoroacetonate derivative readily precipitates in the high millimolar range. However, perfluoration of the ligand did not seem to increase the lipophilicity of the complex, as $Cu(hfacac)_2$ displayed P values similar to the parent acetylacetonate complex. This observation may be rationalized in terms of the stability of the complexes and its influence on the amount of the nondissociated species being investigated. The stability constants ($\log\beta$) of the complexes have been previously reported: $Cu(nta)_2$, 17.42; $Cu(acac)_2$, 14.76; $Cu(tfacac)_2$, 12.20; Cu(hfacac) ($\log K_1$), 2.7 [49, 50]. For the β-diketonates, the electron-withdrawing effect of fluorine atoms decreases the electron density over the donor oxygen atoms, resulting in compounds which are less stable than the parent acetylacetonate. This effect is pronounced in the perfluorated complex $Cu(hfacac)_2$, where simple chemical equilibrium calculations predict that in 50 mM solutions about 20% of the complex will be dissociated as free Cu(II) ions and ligands. In the micromolar range, virtually all the complex is dissociated, which renders $Cu(hfacac)_2$ less interesting for biomedical applications.

Oxidation of the redox-sensitive fluorescent probe DHR has been previously established as a valid indicator of the metal-catalyzed formation of reactive oxygen species in biologically relevant situations (temperature, salinity, pH, and concentrations of both complexes and pro-oxidants). Initially proposed as a means to assess redox-active labile iron in supplements [37, 51], it was soon extended to quantify pools of labile iron in the plasma of iron-overloaded patients [52, 53] and recently to assess the safety of Mn(III) enzyme mimetics [54, 55]. In this study, we observed that the catalysis of DHR oxidation (which is related to the onset of fluorescence) by some of the Cu(II) species is prominent (Table 1; Fig. 1) and occurs to a greater degree than in the case of Fe^{III}(nta), the initial standard for iron-mediated oxidative stress in plasma [52] (data not shown). Also, the susceptibility to peroxide (Table 1; Fig. 2) is markedly

Table 1 Lipophilicity (P) and acceleration of peroxide-assisted oxidation of dihydrorhodamine hydrochloride in the presence of the Cu(II) complexes studied

Complexes	P	Redox activity (min^{-1} (μM H_2O_2)$^{-1}$)
$CuSO_4$	0.010 ± 0.004	0.374 ± 0.022
$Cu(nta)_2$	0.020 ± 0.032	0.014 ± 0.001
$Cu(acac)_2$	0.130 ± 0.025	0.461 ± 0.028
$Cu(tfacac)_2$	0.259 ± 0.012	0.497 ± 0.030
$Cu(hfacac)_2$	0.097 ± 0.007	0.576 ± 0.035

nta nitrilotriacetic acid, *acac* acetylacetone, *tfacac* trifluoroacetylacetone, *hfacac* hexafluoroacetylacetone

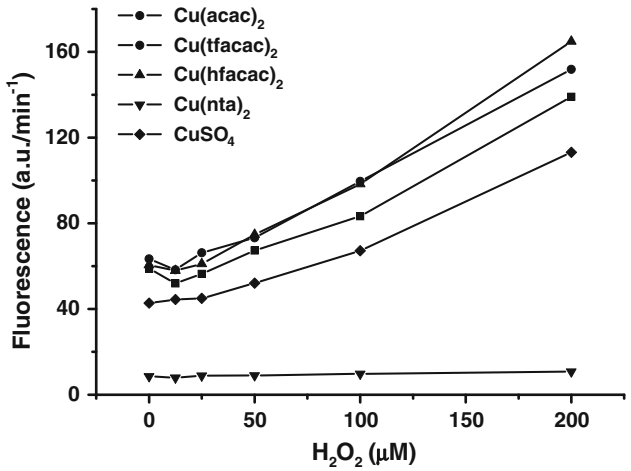


Fig. 1 Oxidation rates for the Cu(II) complexes (5 μM, 37 °C) in the presence of peroxide. *nta* nitrilotriacetic acid

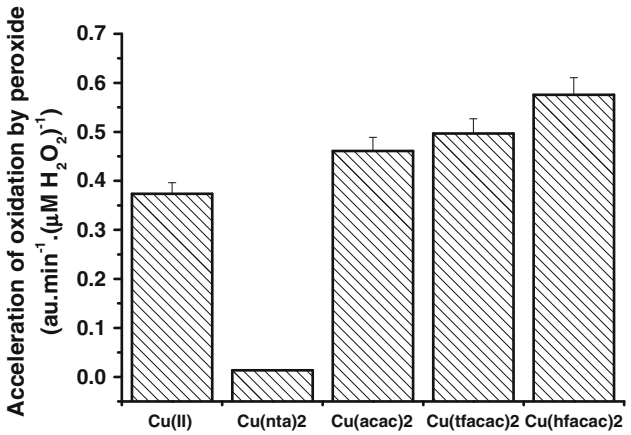


Fig. 2 Acceleration of peroxide-assisted oxidation of dihydrorhodamine hydrochloride in the presence of Cu(II) complexes (5 μM, 37 °C)

different among species, with the β-diketonates being highly active. The combination of $Cu(nta)_2$ with peroxide did not result in a high pro-oxidant activity, which together with its low lipophilicity might be related to its poor performance in the biological tests (see below).

All synthesized complexes displayed antileishmanial activity (Table 2), with $Cu(tfacac)_2$ being the most active compound. Toxicity of all complexes towards *L. amazonensis* promastigotes, except $Cu(acac)_2$, was significantly ($P < 0.001$) higher after 72 h of exposure (Table 2). Interestingly, the EC_{50} values for $Cu(acac)_2$ activity against promastigotes increased after 72 h, indicating that the parasites counterbalance the stressor with increased metabolic activity (which is shown by the MTT assay results), the reasons for which are currently unknown. The free ligands were tested but displayed no activity (data not shown). Considering the four complex species, a trend can be observed (Pearson $r = -0.8810$; $R^2 = 0.776$) of increasing lipophilicity with decreasing EC_{50} values for activity against promastigotes at 72 h, suggesting that this parameter may be involved in the facilitated transport of metal complexes through parasite membranes, as previously discussed [23]. $Cu(tfacac)_2$ displayed the highest lipophilic character as well as high pro-oxidant activity (Fig. 2), and this combination translated into increased toxicity towards *L. amazonensis* promastigotes after 72 h (Table 2), with an EC_{50} value of 43 μM. This result compares favorably with previous findings for other copper fluorocomplexes such as copper bis(trifluorolactate) ($EC_{50/72h} = 167$ μM [23]). We determined the ability of $Cu(trifluorolactate)_2$ to accelerate the peroxide-induced oxidation of DHR in the same setup as that described above and found that it is comparable (0.568 min^{-1} $[H_2O_2]^{-1}$) to that of $Cu(tfacac)_2$ (see Table 1). However, tfacac is predicted to be more lipophilic than trifluorolactic acid (log*P* of 1.935 and 0.784, respectively [56]), which was reflected in a rather low *P* (0.014) for $Cu(trifluorolactate)_2$. These observations suggest that a combination of high lipophilicity and high pro-oxidant activity is desirable in order to produce more effective copper antileishmanials. High toxicity to Vero cells was detected for all compounds tested (Table 2), demonstrating that the antileishmanial activity of these compounds lacks specificity. This should be taken into account in the design of more specific compounds.

Table 2 EC_{50} (μM ± Standard deviation) values for activity of the Cu(II) complexes studied against promastigotes of *Leishmania amazonensis* and Vero cells

Complexes	Promastigotes			Vero cells		
	24 h	48 h	72 h	24 h	48 h	72 h
$CuSO_4$	129 ± 4	96 ± 11	58 ± 16	142 ± 5	109 ± 15	31 ± 8
$Cu(nta)_2$	263 ± 2	293 ± 3	122.0 ± 0.3	342 ± 47	137 ± 2	13 ± 2
$Cu(acac)_2$	31.0 ± 0.2	84 ± 2	94 ± 3	83 ± 5	19.0 ± 0.5	15 ± 14
$Cu(tfacac)_2$	68 ± 7	57.7 ± 0.2	43.0 ± 0.2	74 ± 4	31 ± 6	25 ± 11
$Cu(hfacac)_2$	135 ± 2	124 ± 4	68 ± 3	127 ± 47	39 ± 9	12.6 ± 0.2

Table 3 Activity of *Typanosoma cruzi* trypanothione reductase in the presence of inhibitors

Complexes	Activity (%)
Control	100
Clomipramine	40.7
$CuSO_4$	53.9
$Cu(nta)_2$	45.8
$Cu(acac)_2$	42.3
$Cu(tfacac)_2$	7.01
$Cu(hfacac)_2$	37.5

Clomipramine, 10 μM; copper species, 50 μM (metal-based)

Additional evidence of the importance of the lipophilic character of the complex for antiparasitic activity came from the inhibition tests with trypanosomatid-specific TR (Table 3), where it was found that $Cu(tfacac)_2$ was the most potent inhibitor of this parasite-specific enzyme. The active site of TR is embedded in a lipophilic pocket, and tricyclic antidepressants such as clomipramine dock in a similar way as the actual TS_2 substrate, thereby stopping enzyme functioning [57]. The hydrophobic moiety of the TR active site is therefore more suited to accommodate similar-polarity compounds such as the bis(trifluoroacetato) complex. These results suggest that the rational design of antileishmanial metallodrugs could consider the docking capacity of the complexes of the highly lipophilic environment of TR.

Conclusions

It was determined that the integration of high lipophilicity (provided by the appropriate choice of ligands) and peroxide-assisted pro-oxidant activity is effective in the design of Cu(II) antileishmanial metallodrugs. This concept may be extended to other inorganic compounds in which the metal center is able to promote the generation of reactive species when in the presence of endogenous peroxide, thus leading to redox disruption of the parasite. Also, lipophilicity modulation by the ligands is advantageous in the selective inhibition of parasite TR. Selectivity of these compounds has yet to be achieved to reduce the toxicity of the complexes towards nontarget cells.

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