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Original paper

In silico and *in vitro* inhibitory potential of an organometallic Cu (II) complex on *Leishmania major* stages

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ABSTRACT. Leishmaniosis results in a serious complication, principally in the tropical and subtropical areas. Metal-cored complexes, like meglumine antimoniate (MA) have proven antileishmanial activity. Similarly, in this research, we investigated the effects of Cu (II) dimethoxy bipyridine (CuDMOBP) against *Leishmania major* stages *in silico* and *in vitro*. Molecular docking analysis was carried out on the complex and a protozoan metacaspase. The complex's anti-promastigote and its cytotoxicity towards macrophages were assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method to calculate relative Inhibitory Concentration 50% (IC_{50}), Cytotoxic Concentration 50% (CC_{50}), and Selectivity Index (SI). Expression of TNF-α and IL-10 in intracellular amastigotes and induction of apoptosis was also investigated using quantitative real-time PCR. The complex interacted effectively with four amino acid residues including lysine (Lys171), histidine (His193), arginine (Arg44 and Arg243) of the targeted metacaspase. This indicates a potential affinity between the target macromolecule and the complex. MTT results showed significant in vitro inhibitory effects against promastigotes. Reduction in cellular expression of IL-10 and TNF-α was also significant, $p<0.05$ and $p<0.005$, respectively. CuDMOBP showed powerful *in vitro* anti-leishmanial activity and could be introduced as a new leishmanicidal candidate.

Keywords: *Leishmania major*, Cu (II) dimethoxy bipyridine, TNF-α, IL-10, cytotoxicity

Introduction

Cutaneous leishmaniosis (CL) as the most common presentation of leishmaniosis has a global distribution. The disease is primarily caused by the protozoan parasites, *Leishmania major* and *L. tropica* in the Afro-Eurasia. Iran, Afghanistan, Brazil, Saudi Arabia, Peru and Syria [1] account for ninety percent of the world CL annual cases. The clinical presentations of *Leishmania* infections may extend from a localized single lesion to multiple cutaneous lesions and vary from mucosal to systemic visceral involvement which could be potentially fatal [2]. Although, CL, in most cases, is a nonfatal and self-healing disease, complete cure can take months to years [3]. Additionally, secondary infection, functional impairment, development of disfiguring permanent scars, parasite dissemination to mucous membranes or relapse may happen throughout the recovery period [4,5]. Therefore, the main purposes in CL therapy are to reduce the recovery period and prevent the parasite propagation by improving the host's ability to heal the lesions and to kill the causative agent as well [6,7].

Current medication for leishmaniosis including pentavalent antimonials (e.g. Glucantime® or meglumine antimoniate (MA)) are limited by their

side effects, toxicity, and drug resistance [4]. Other second-line drugs such as amphotericin-B, paromomycin and miltefosine are also disadvantageous due to their high price, resistance and insufficient effects [7,8].

Although copper is involved in many important biological processes [9], compounds containing Cu have long been considered as potent inducers of oxidative stress or damage both *in vitro* and *in vivo* [10]. Metal complexes, on the other hand, are able to inhibit parasite-specific enzymes such as cysteine proteases [11], aquaporin water channels, aquaglyceroporin [12], peptidases [13], and oxidoreductases [14,15]. Literature indicates that metallocomplexes may be dominantly suited to reach the parasite. In organometallic complexes, the metal ion is coordinated by organic moieties containing heteroatoms like nitrogen and oxygen and other electron pair donor atoms. These organic ligands coordinate with metal ions through donation of their electron pairs. Through this mode of action in part, they may contribute to the cellular uptake as well as the biological properties of organometallic complexes [16]. Intercalation of copper containing compounds with DNA has also been regarded as an effective approach in the design of bioactive Cu (II) complexes [17]. Screening of antileishmanial drug starts de facto with *in vitro* cytotoxicity studies against promastigotes with expansions to *in silico* and *in vivo* trials.

By default, the crystal structures of macromolecules are chosen from the protein data bank (PDB) for such analyses. An array of the established leishmanial protein drug targets has been subjected to molecular docking studies [18]. Caspases are the center of focus as proteins playing a central role in programmed cell death (PCD) in mammalian cells. Metacaspases replace caspases in protozoa, plants and yeasts in this regard. These enzymes could be chosen as targets for docking studies [19].

Alterations in the cytokine release profile of macrophages in response to pathogen contamination have well been documented [20]. It has been observed that intracellular amastigotes of *Leishmania* are able to trigger the release of interleukin-10 (IL-10) and tumor necrosis factor-alpha (TNF-α) and, for which the parasite's zinc metalloprotease GP63 has been nominated [21]. TNF-α as a biomarker of type 1 T helper cells (Th1) line, is an essential component in *L. major* cutaneous infections, contributing to control of the inflammatory reactions and parasite killing [6]. On the other hand, IL-10 as the major index of type 2 T helper cells (Th2) set represents the major regulatory cytokine in leishmaniosis and induces parasite persistence and dissemination [22,23].

In this work, we embarked on evaluation of the affinity of CuDMOBP *in silico* toward a parasitic metacaspase available in PDB. Then *in vitro* studies against *L. major* promastigotes using MTT. Furthermore, the complex's mode of action was explored by flow cytometric analysis to elucidate its apoptotic profile

Materials and Methods

Cu (II) complex

The complex (CuDMBOP) contained two 2,2'-dimethoxy-4,4'-bipyridines ligands and two PF_6^- as counter ions and was synthesized and characterized as previously described [16]. In brief, $CuCl_2.H_2O$ and dimethoxybpy were dissolved in ethanol and stirred for 5 h at room temperature. After addition of a saturated aqueous NH_4PF_6 solution, the blue product, $[Cu(dimethoxybpy)_2]$ $(PF_6)_2$, was collected by suction filtration, washed with cold water and ether, and air-dried. Recrystallization by slow evaporation of an acetonitrile/dioxane solution of the complex gave shiny blue single crystals suitable for crystallography. CuDMOBP was dispersed in aqua using a probe sonicator prior to use. A 10 mM suspension of the compound in water was as stock solution.

Docking studies

There are only nine structures containing crystallographic data on metacaspases in PDB, of which the closest match to leishmanial metacaspase was found to be the metacaspase II (PDB ID: 4AF8) of *Trypanosoma brucei brucei*, with 62.35% similarity. The Molecular Operating Environment (MOE 2015.10) was used for the molecular docking procedure of the complex [24]. The complex's structure was prepared via an energy minimization protocol to reduce the clashing parameters and to optimize the geometrical bonds. Initially, the compound underwent a typical refinement of its structure by the QuickPrep toolbox of the software to attain a root mean square (RMS) gradient of below 0.001 kcal/mol/Å. Protonate three-dimensional (3D) was utilized for the protonation of the system. Next, a final optimization step was applied using the energy minimize module for the preparation of the ultimate structure. At this

stage, the all-atom Amber 10: EHT force-field (developed for preparation of both small-molecules and proteins) was used for fixing the charge of the complex and conformational optimization. The structure of the *Trypanosoma brucei brucei* metacaspase was extracted from the crystallographic protein data bank (PDB) structure with 1.4 Å resolution, PDB ID: 4AF8 [19]. The UniProt database only displays a putative entry for *Leishmania major* metacaspase, and therefore the docking procedure was executed using 4AF8 structure. The enzyme structure was optimized using the QuickPrep and energy minimize module with a similar approach used for the preparation of the ligand complexes.

Molecular docking was carried out by virtue of a whole grid on protein structure (blind docking). Although blind docking may result in lower accuracy than an exact-grid based molecular docking, we utilized it due to our lack of prior knowledge on how various ligands dock to binding pockets of the enzyme. In the compute tab of the MOE software, the DOCK module was utilized for molecular docking. The initial scores were computed with triangle matcher by London dG scores with 50 poses, and the final scores were calculated via London dG scores and induced fit refinement approach in the MOE software. PyMol visualization software was used for the anticipation of 3D binding patterns of the ligand and its receptor protein.

Parasite culture and treatment

Promastigotes of *L. major* (MRHO/IR/75/mash2, the standard strain) were first grown at 5×10^5 cells/ml in Roswell Park Memorial Institute 1640 (RPMI 1640) medium containing 100 µg/ml of streptomycin, 100 U/ml of penicillin and 10% (v/v) inactivated fetal bovine serum (FBS) at 24±1°C (pH 7.2). Then, the promastigotes in logarithmic growth phase were added to round-bottomed 96-well culture plates at 10^5 cells/100 µl/well and treated in triplicate with 10 µl of gradient concentrations (0, 50, 100, 200, 400, and 800 µg/ml) of the complex and control drug separately. Initial cell numbers were calculated directly in the Neubauer chamber [25].

Treatment and cell proliferation measurements

After 72 h of incubation, 10 µl of syringe filtered MTT stock solution (5 mg/ml) in PBS was added to all wells and incubated at 24±1°C for 3 h. Afterwards, parasite cell-suspensions were centrifuged, the supernatant was removed and DMSO (100 µl) was added to cell sediments and incubated for 15 min to dissolve formazan crystals. By an enzyme-linked immunosorbent assay (ELISA) reader, the absorbance was read at 570 nm. Intensity of the absorbance due to the reduction of MTT to formazan was taken proportional to the number of viable cells and inversely proportional to the compounds' antipromastigote effect on *L. major* [26].

Flow cytometric analysis

To determine whether CuDMOBP is a preferential inducer of apoptosis, the Apoptosis Detection Kit (Mab Tag's, Germany) was applied as instructed by the manufacturer. Briefly, in a microtube containing 10^6 logarithmic phase promastigotes in RPMI 1640 medium (1 ml) a dilution of CuDMOBP (100 µg/ml) was added. The sample was incubated at 24±1°C for 72 h under 5% CO_2. Then, the microtube was washed three times with PBS, followed by suspension in 100 µl of binding buffer (1×). To this, 5 µl of annexin V-FITC and PI stain was added and incubated in the dark at ambient temperature for 20 min. The final analysis was made by flow cytometry (BD FACSCalibur, USA) after addition of 400 µl binding buffer (1×) within 15 min. Cell Quest software (FloMax Software) was used for the analysis [27].

Quantitative real-time PCR (qPCR)
Intramacrophage amastigote growth

Murine macrophages (J774 A.1) were let to adhere at 10^6 cell/well to 6-well cell culture plates for 2 h at 37°C and 5% CO_2. Stationary phase *L. major* promastigotes were then added to these macrophages at a ratio of 10:1. The combination was incubated for 24 h at 37°C and 5% CO_2 to let amastigotes evolve prior to treatments with applied concentrations of the complex for 72 h at the same conditions.

RNA isolation and analysis

Total RNA of murine macrophages infected with amastigotes of *L. major* as the parasites clinical stage, was obtained using EZ-10 Spin Column Total RNA Miniprep Kit (Bio Basic Inc., Canada) according to the instruction's manual. Absorbance ratio at 260 and 280 nm (A260/280) on a ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) was used to determine the purity and

Table 1. Primers used for real-time PCR

Gene	Forward sequence (5'-3')	Reverse sequence (5'-3')	Product size (bp)
IL-10	CTTACTGACTGGCATGAGGATCA	GCAGCTCTAGGAGCATGTGC	101
TNF-α	TCTCCTTCCTGATCGTGGCA	AGGTCCCTGGGGAACTCTTC	94
GAPDH	AGCTTCGGCACATATTTCATCTG	CGTTCACTCCCATGACAAACA	89

quantity of the extracted RNA.

Real-time quantitative PCR

A sum of 500 ng mRNA was reverse-transcribed to cDNA, utilizing Revert Aid M-MuLV reverse transcriptase and random hexamer primers (both from Fermentas, Vilnius, Lithuania). Quantitative RT-PCR of target cDNAs was conducted on a Rotor Gene 6000, Corbett, Real Time PCR Machine (QIAGEN, United States). Table 1 shows the sequence of primers as well as the PCR products expected for GAPDH and cytokine genes. The PCR reactions were done in 10 µl reaction volumes possessing 5 µl 2X SYBR Green Supermix SYBR® Premix Ex TaqTM (Takara Bio Inc., Otsu, Japan), 250 nmol of forward and reverse primers, and 1 µl cDNA template diluted with RNase-free H_2O.

The two-step of PCR-polymerization conditions

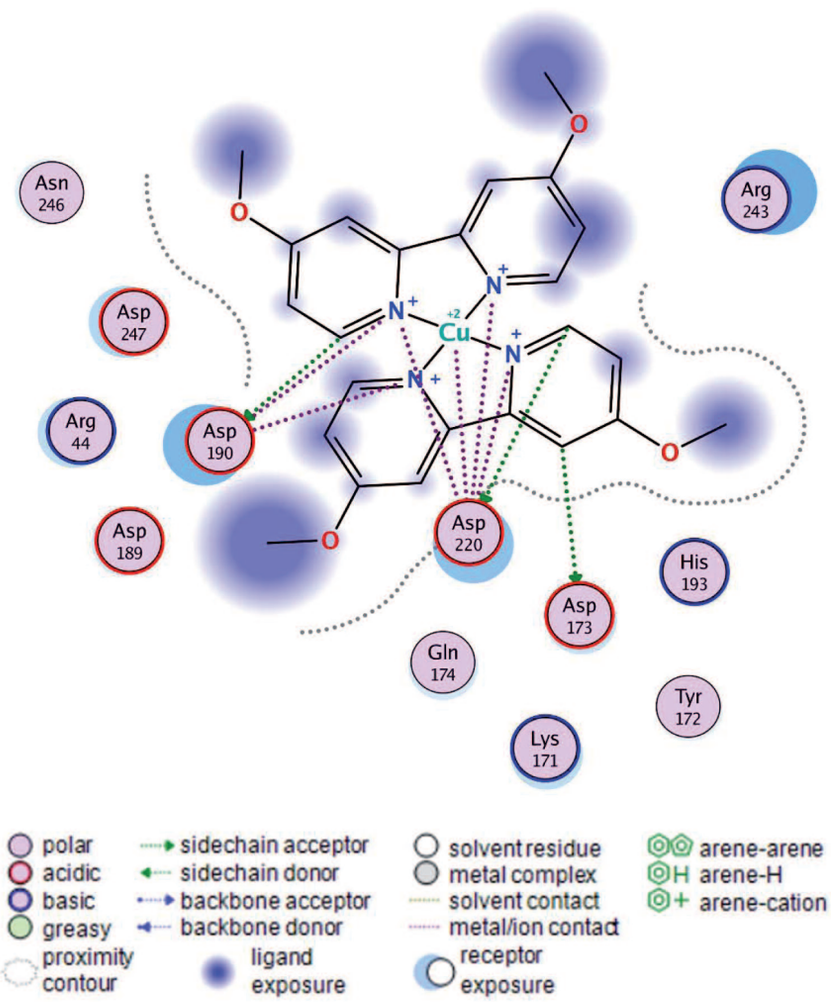

Figure 1. The interactions and binding orientations of the ligand complex with trypanosomal metacaspase (4AF8)

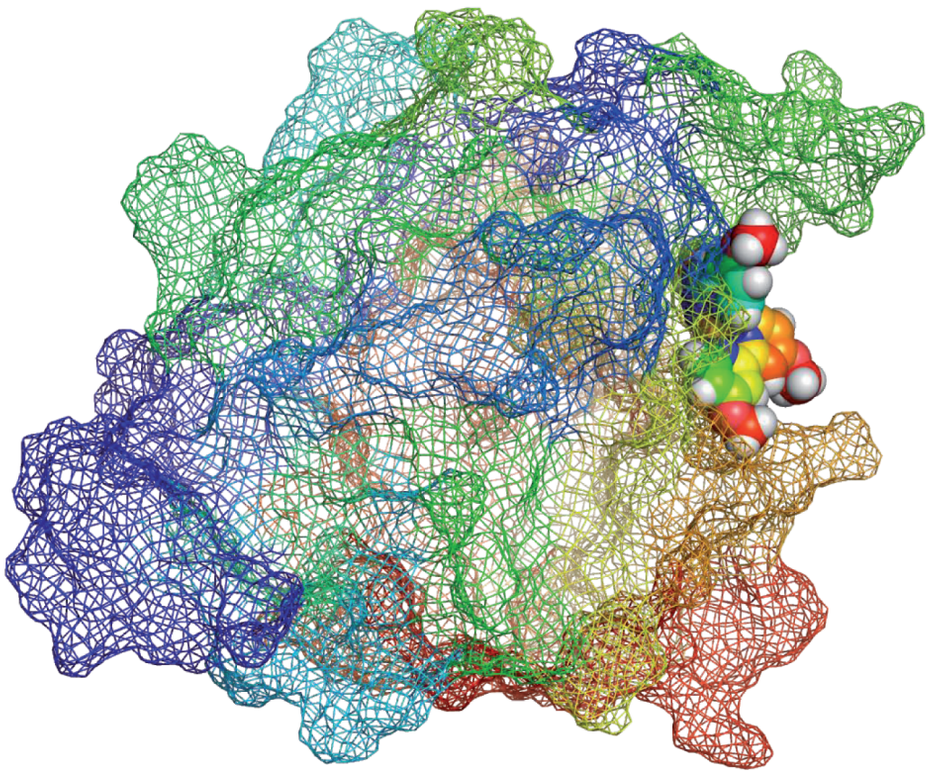

Figure 2. The three-dimensional (3D) structure of Cu (II) dimethoxy bipyridine (CuDMOBP) docked in the binding site of 4AF8, using PyMol visualization software

included 95°C (30 s) and then 40 cycles of 95°C (5 s) and 60°C (30 s). Each series of reactions included a negative control (i.e., water only) and RT control (samples containing not reverse transcribed RNA). For each gene, PCR reactions were performed in duplicates. The data of PCR were optimized to the levels of GAPDH gene as reference genes. Δ^{CT} calculated according to the formula: [Δ^{CT} = CT $_{(target)}$ - CT $_{(control)}$]. Gene expression levels were measured by $2^{-\Delta Ct}$ method. Fold increase (FI) was analyzed based on the comparative threshold method ($2^{-\Delta\Delta CT}$) [26].

Statistical analysis

The data were analyzed using the SPSS version 20.0 (Chicago, IL, USA). Cell viability data were statistically analyzed by two-way ANOVA and T-test. The GRAPHPAD PRISM 6 (GraphPad Software Inc, CA, USA) was used to analyze the mean $2^{-\Delta\Delta CT}$ for treatment and control of each cytokine expression measurement.

Results

The molecular docking results suggest that several aspartate residues of the metacaspase structure are key players in maintaining the CuDMOBP ligand complex in the enzyme binding pocket (Figs. 1 and 2). Cu^{2+} in the complex structure and quaternary ammonium atoms were involved in the ionic interactions with these aspartate residues in metacaspase structure. In the complex free structure, the aspartate residues were stabilized by the adjacent positively charged amino acids like lysine (Lys171), histidine (His193), arginine (Arg44 and Arg243). The most critical amino acid residue in the interactions with the ligand molecule was Asp220, which formed ionic interactions with three quaternary nitrogen and the Cu^{2+} atom. Therefore, the binding of the positively charged complex structures create several charged layers in the structure of the metacaspase-ligand complex, and these multilayers contribute to the stability of the complex.

In vitro anti-promastigote effect of the complex was carried out by MTT. The test compound and the positive control drug inhibited the growth of *L. major* promastigotes in all incubation time settings of 72 h (Fig. 3). The 50% inhibitory concentrations (IC_{50}) of the three compounds are presented in table 2. At 50 µg/ml and 24 h incubation, CuDMOBP did not perform significantly different from the control

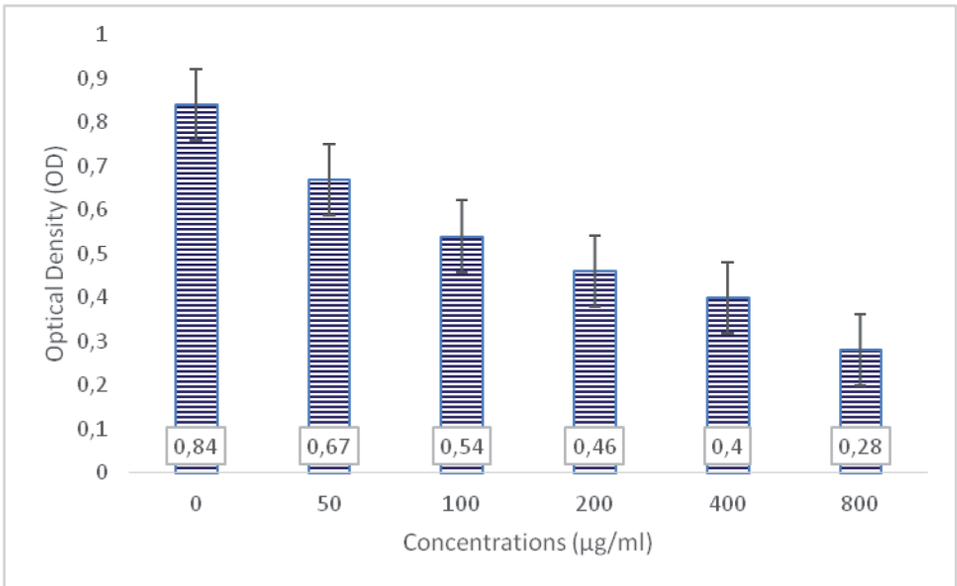

Figure 3. Comparison of optical density (OD) between untreated cells and gradient concentrations of Cu (II) dimethoxy bipyridine (CuDMOBP) (800, 400, 200, 200, 100,50 µg/ml) on the susceptibility of *Leishmania major* promastigotes by MTT colorimetric assay

drug. However, with increasing concentrations and incubation time, the OD values significantly decreased and inhibitory percentage increased ($P<0.05$).

The percentages of cells at early apoptosis (annexin-V positive), late apoptosis (annexin-V and PI positive), necrosis cells (propidium iodide positive) and living cells (annexin-V and PI negative) with CuDMOBP (100µg/ml) were determined following a 72-h culture treated with the complex and the control drug. The apoptotic cell populations were 29.45 (Fig. 4).

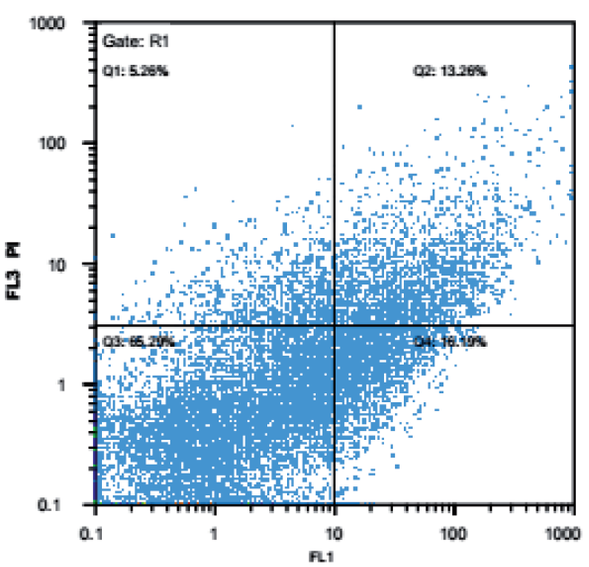

Figure 4. Flow cytometry results illustrating apoptotic cells after treatment at 100 µg/ml of Cu (II) dimethoxy bipyridine (CuDMOBP) for 72 h

Comparison of the mean $2^{-\Delta\Delta CT}$ between untreated cells and those cells treated with CuDMOBP showed changes in levels of TNF-α and IL-10 mRNAs. The mean fold increase (FI±SE) when treated at 100 µg/ml of CuDMOBP for TNF-α and IL-10 was 0.07±0.007 ($P<0.05$) and 0.27±0.03 ($P<0.005$), respectively. These two factors were 0.11±0.001 ($P<0.05$) and 0.01±0.0001 ($P<0.01$) when cells were incubated with MA (Fig. 5).

Discussion

This research was an effort to investigate the potentials of a copper complex combined with two dimethoxy bipyridine ligands as a new drug candidate against promastigotes of *L. major*, the causative agent of zoonotic cutaneous leishmaniasis, a necessity highlighted by the disadvantages of current treatments [28]. The complex's *in silico* affinity towards a protozoan metacaspase, its *in vitro* inhibitory effects against *L. major* promastigotes was studied. Also, the cytotoxicity of the complex towards murine macrophages as parasite's host, and its ability to modulate the expression of two cytokines (Il-10 and TNF-α) were investigated.

Copper compounds have been considered for long time as broad-spectrum biocides, owing to their ability of inducing cell membrane and protein

Table 2. *In vitro* antipromastigote activity of Cu (II) dimethoxy bipyridine (CuDMOBP) against *L. major* promastigotes after 72 h of incubation

Drug/Values	IC_{50}	CC_{50}	SI
(µg/ml)	Promastigote	Macrophage	
CuDMOBP	141.26	141.41	1.001

damages, deactivating reduced glutathione and strongly bind to nucleic acids [29]. Incorporation of copper in complexes with polycyclic organic ligands has been proposed to hinder the reactivity of the free metal and its delivery to the aimed organisms/biological targets [28].

In silico studies indicate that the activity of the trypanosomal metacaspase could be inhibited by the use of tetrapyridine structures coordinated with metallic atoms to conform a positively charged complex capable of tight binding to the metacaspase structure. Concisely, the two aspartate residues appeared to be essential in binding of the complex, Asp190 and Asp220. Asp173 aids in the stability of the ligand in the binding site of metacaspase for methoxy complex. Neither of the oxygen groups in the four methoxy functional groups contributed to the stabilization of the ligand and this enhances the validity of the mentioned premise.

From a 3D perspective, the complex's structure satisfactorily fits in the 4AF8 metacaspase structure. Therefore, appropriate fitting of the ligand structure with London forces supplies further stability in the ligand-receptor structure (Fig. 2). Moreover, based on the high number of aspartate residues and their avid interactions with the positively charged complexes, it is possible to speculate that the complex imposes its anti-leishmanial activity, at least partly by inhibiting the activity of metacaspases and inducing cell death.

There were no leishmanial metacaspase structures in the protein data bank (PDB) at the time of performing this project. Therefore, a trypanosomal metacaspase (PDB id: 4AF8) belonging to a close protozoan relative was chosen for molecular docking studies. Absolute application of the findings to crystal structures of leishmanial metacaspase hence remains to be investigated when such structures published.

Consistent with our findings, investigation on a series of Cu complexes containing fluorinated α-hydroxycarboxylates against *L. major* promastigotes has revealed that the more hydrophobic and redox-active compounds the higher their toxicity [15]. Reaction of Cu with endogenous peroxide species (O-O, R-O-OH, R^1-O-O-R^2, and R^1-CO-O-O-R^2,

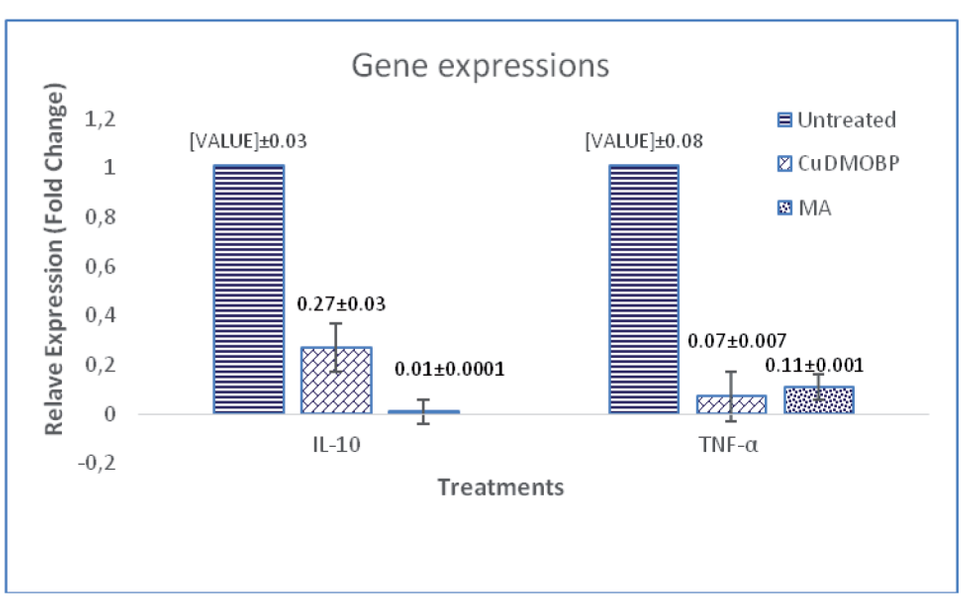

Figure 5. Gene expression profiles of IL-10 and TNF-α in macrophages treated with Cu (II) dimethoxy bipyridine (CuDMOBP) and meglumine antimoniate (MA) as compared to untreated cells (Error bars indicate standard error (SE) ($p<0.05$)

where $R^1$and R^2 = hydrocarbons) results in the generation of reactive oxygen species (ROS) and promastigotes killing, have been proposed to mediate the effect [17]. Méndez-Arriaga et al. [28] studied the *in vitro* antiparasitic activity of Cu complexes ($[Cu_2(\mu\text{-}7atp)_4Cl_2]C_{l2}\cdot 4H_2O$ (1) and $[Cu_2(\mu\text{-}7atp)_4(H_2O)_2]$ $(NO_3)_4\cdot H_2O$(2)) against three different strains of *Leishmania* spp. and *Trypanosoma cruzi*, reporting a higher efficacy than the commercial reference drugs. Singh et al. [30] demonstrated that copper salicylaldoxime (CuSAL), has potent anti-leishmanial activity, selectively interacting with LdTOPILS; and is safe for humans. Therefore, this compound might represent a highly promising candidate for rational chemotherapy approaches to control human leishmaniosis.

Induction of TNF-α and decrease in IL-10 release from macrophages are two phenomena correlated to parasite engulfment macrophages. High levels of TNF-α have been found in the sera of pretreatment CL patients [31,32]. This cytokine has been shown to mediate the production of NO and the killing of *L. major* parasites [32]. Interestingly, cultured cells are only able to produce TNF-α in response to alive parasites, but not parasite antigens [33]. Accordingly, the decline in the levels of TNF-α expression is attributed to a reduction in population of alive intra-macrophage amastigotes. Similarly, reductions of IL-10 expressioin may be the result of decline in parasite population. We observed reductions in the expression of both TNF-α and IL-10 in response to the treatment of amastigote-contaminated macrophages with the CuDMOBP and MA in comparison to the untreated macrophages. Findings of several studies agree with the results of our study [6,25].

It is however noteworthy that these reductions did not go in line with both drugs. While CuDMOBP reduced the expression of TNF-α more than MA did, MA suppressed the expression of IL-10 more in turn.

Finally, the inevitable selection of a trypanosomal metacaspase as the host molecule for docking studies of the complex CuDMOBP in the absence of a suitable leishmanial target, resulted in probing interactions between these two molecules. This could motivate future research to test the complexes against *Trypanosoma* species as well.

In conclusion, the present results showed that CuDMOBP indicated a powerful *in vitro* anti-leishmanial activity. Hence, the *in silico* and *in vitro* findings of the present study demonstrated that CuDMOBP could be a potentiall candidate drug to be used in murine model and/or human settings in future control programs.

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