

Inhibitory Effect of Silver Nanoparticles on Trypanothione Reductase Activity and *Leishmania infantum* Proliferation

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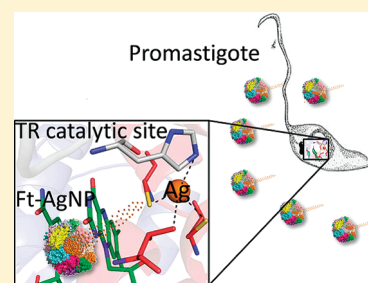
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 Supporting Information

ABSTRACT: In *Leishmania* the glutathione/glutathione reductase eukaryotic redox system is replaced by the unique trypanothione/trypanothione reductase (TR) system. *In vitro*, silver is a more effective TR inhibitor than antimony, the first line drug against leishmaniasis in most endemic countries, and its mechanism of inhibition is similar to that of Sb(III). In particular, silver binds with high affinity to the catalytic triad Cys52, Cys57, and His461', thereby inhibiting TR. Here, Ag(0) activity was tested on the promastigote and amastigote stages of *Leishmania infantum* using a drug-delivery system consisting in Ag(0) nanoparticles encapsulated by ferritin molecules (Pft-AgNPs). These were able to induce an antiproliferative effect on the parasites at metal concentrations lower than those used with antimony.

KEYWORDS: Trypanothione reductase, *Leishmania*, silver, nanoparticles, drug delivery



Leishmaniasis affects at present 12 million people worldwide.¹ Because effective vaccines are not yet available, the chemotherapy remains the only treatment option for controlling the infection. Except for the recent encouraging advances in chemotherapy obtained with amphotericin B and its new liposomal formulations, miltefosine and paromomycin, the treatment modalities for leishmaniasis infections mostly rely on antimony-based drugs, which date back over 60 years and suffer from poor efficacy, high toxicity, and increasing resistance.² The *Leishmania* life cycle involves two principal morphological stages, promastigote and amastigote. The first one develops within an insect vector, a phlebotomine sand-fly; the second one infects the macrophages of vertebrate hosts, which produce considerable amounts of H₂O₂ to eliminate intruding parasites. The search for urgently needed drugs against *Leishmania* parasites is focusing on metabolic pathways vital for the amastigote parasite and distinct from the analogous metabolism in the mammalian host. In this respect, the trypanothione metabolism enzymes are considered as promising targets of new drugs against leishmaniasis.

The trypanothione (T(SH)₂) is synthesized from glutathione and spermidine by the trypanothione synthetase (TryS) and is kept reduced by the trypanothione reductase (TR). Trypanothione participates in crucial thiol–disulfide exchange reactions and serves as electron donor in several metabolic pathways, from synthesis of DNA precursors to oxidant detoxification. The T(SH)₂/TR system replaces many of the antioxidant and metabolic functions of the glutathione/glutathione reductase (GR) and thioredoxin/thioredoxin reductase (TrxR) systems present

in other organisms and therefore is necessary for the parasite survival.^{3–5} Sb(V), reduced inside the amastigote to Sb(III),⁶ interferes *in vivo* with the T(SH)₂ metabolism by inducing rapid efflux of intracellular T(SH)₂ and inhibiting TR in intact cells.⁷ We disclosed the molecular mechanism of antimonial inhibition of TR by structural analysis of reduced TR in complex with NADPH and Sb(III).⁸ This structure revealed that all residues of the catalytic triad, i.e. Cys52, Cys57, and His461', are engaged in complex formation with Sb(III).

Several metal complexes have been tested on a variety of trypanosomatids and resulted to be active on different molecular targets.^{9–11} For example, (2,2':6',2'' terpyridine)platinum(II) complexes have been found to be effective on *Leishmania donovani*, *Trypanosoma cruzi*, and *Trypanosoma brucei* and to have a dual action: the terpyridine moiety interacts with DNA, whereas the metal binds to the thiol containing enzymes.⁹ Gold(I) triphenylphosphine complex has potent antiproliferative effects on *Leishmania* spp. and *Trypanosoma cruzi*, due to the NADH fumarate reductase inhibition.¹⁰ Silver polypyridyl complexes are biologically active against *Leishmania mexicana*, where they interact with DNA.¹¹ Silver has also been shown to have antibacterial action at very low concentrations, since it reacts very easily with protein carboxylates, hydroxyls, and thiols. Thus, silver compounds are used as antibacterial agents.¹²

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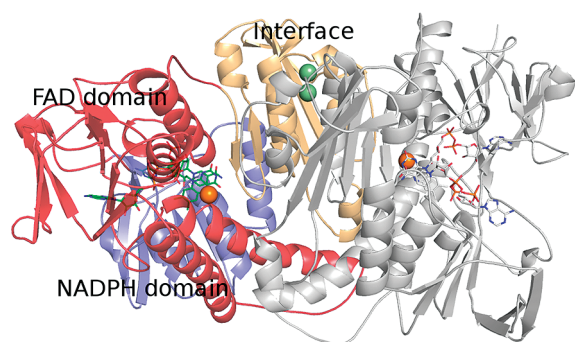


Figure 1. Structure of reduced TR in complex with NADPH and silver (PDB code 2X50). Silver atoms in the catalytic sites are represented as orange spheres whereas silver atoms at the dimeric interface are represented as light green spheres. NADPH and FAD molecules are represented as sticks. Figure generated by PyMol.¹⁹

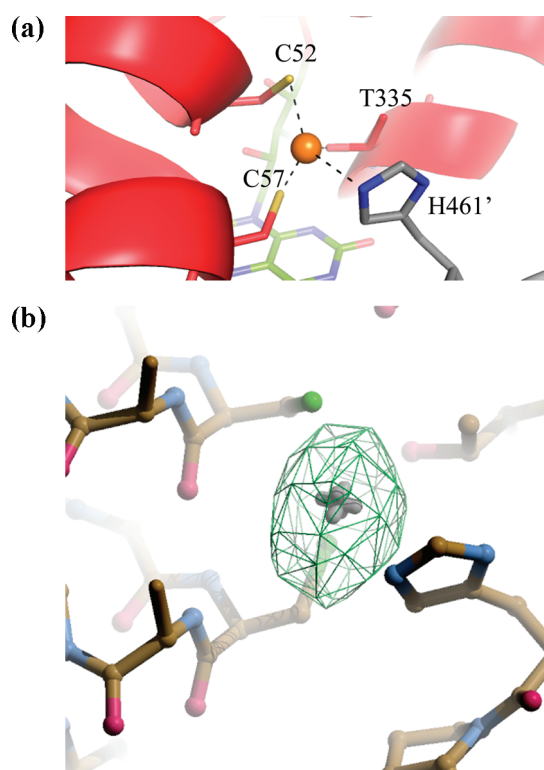


Figure 2. (a) Close view of the catalytic site of TR, with bound silver represented as an orange sphere. The two subunits in the asymmetric unit are colored in red and in gray respectively; the FAD molecule is colored in green. C52, C57, T335, and H461' are represented as sticks. (b) Stick representation of the catalytic site and the Fourier difference map $F_o - F_c$ (in green) contoured at 6σ . Figure generated by PyMol¹⁹ and Coot.²⁰

Recently, our group demonstrated the ability of *Pyrococcus furiosus* ferritin (PfFt) to act as a protein template for the production of Ag(0) nanoparticles encapsulated by ferritin molecules (AgNPs).¹³ The AgNPs are characterized by a narrow size distribution (2.1 ± 0.4 nm), high stability in water solution at millimolar particle concentrations, and high thermal stability. The high solubility and stability in water are two essential properties of the silver nanoparticles (NPs), to make them suitable for biomedical applications.

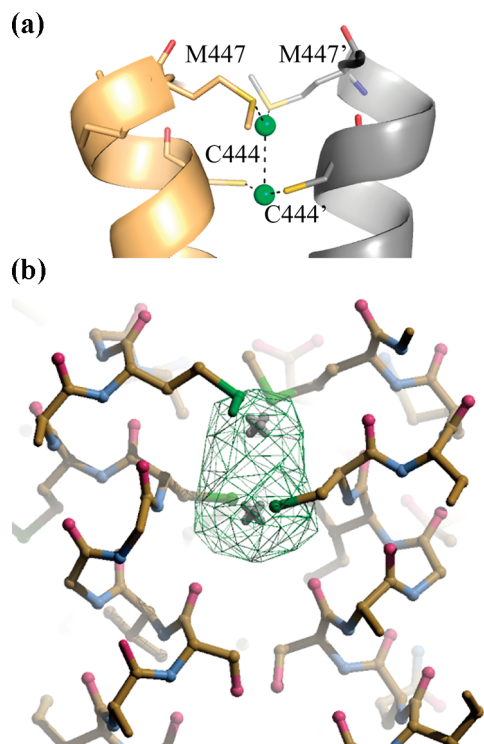


Figure 3. (a) Interface binding site of silver atoms to TR with bound silver represented as green spheres. The two interface domains in the asymmetric unit are colored in yellow-orange and in gray, respectively; C444, C444', M447, and M447' are represented as sticks. Each Ag atom is coordinated to two residues of each subunit: C444 (SG–Ag = 1.9 Å) and M447 (SD–Ag = 2.2 Å), and C444' (SG–Ag = 1.8 Å) and M447' (SD–Ag = 2.4 Å), respectively. (b) Stick representation of the interface silver binding site and the Fourier difference map $F_o - F_c$ (in green) contoured at 6σ . Figure generated by PyMol¹⁹ and Coot.²⁰

In the present work, we investigated the inhibitory effect of silver on TR activity. Moreover, we determined and analyzed the X-ray structure of the complex between Ag(0) and TR in order to understand the molecular basis of protein inhibition. Finally, we measured the inhibitory effect of silver NPs in *in vitro* models of parasite growth and infectivity. The high solubility of PfFt-AgNPs and the fact that ferritins are known to be phagocitized by the macrophages, where the *Leishmania* parasites multiply,^{14,15} were exploited to assess the potential application of PfFt-AgNPs as antileishmania agent.

Experiments for calculation of the K_i of Ag(I) and Ag(0) were carried out by adding different concentrations of Ag(I) to a solution containing oxidized TR and 100 μ M NADPH. After starting the reaction by addition of oxidized trypanothione (TS₂), the absorbance at 340 nm decreases, indicating NADPH oxidation. The curve displaying trypanothione reduction by TR in the presence of Ag(I) is biphasic, since Ag(I) ($E_0 = 0.80$ V) is reduced to Ag(0) within a few minutes by NADPH ($E_0 = 0.32$ V). In order to separate the contribution of Ag(I) and Ag(0) to TR inhibition, TR and TS₂ were added to the reaction mixture after 3 min, the time necessary for a complete reduction of Ag(I) by NADPH. The experimental data were fitted using the Michaelis–Menten equation. These experiments show that TR is strongly inhibited *in vitro* by silver in both oxidized and reduced form. In fact, the inhibition constant measured for Sb(III) is 1.5 μ M,⁸ whereas the inhibition constants calculated for Ag(0)

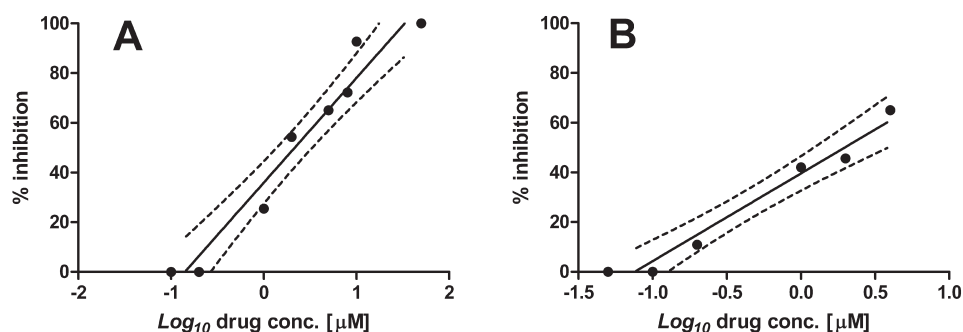


Figure 4. % Inhibition calculated on *L. infantum* stages after treatment with various concentrations of AgNPs. The data are expressed as mean \pm error of two independent experiments. A: promastigotes inhibition. B: amastigotes inhibition.

and Ag(I) are 500 ± 200 nM and 50 ± 10 nM, respectively (see Fig. S2 of the Supporting Information).

The ability of silver to inhibit TR is explained at the molecular level by the crystal structure of reduced *L. infantum* TR in complex with NADPH and silver solved at 3.3 Å resolution (see Supporting Information) (Figure 1). Indeed, the difference electron density map $F_o - F_c$ shows the presence of a strong peak in both monomers, corresponding to the Sb(III) binding site, that has been assigned to a silver atom with an occupancy of 80% in both subunits (Figure 2). Ag(0) binds, as Sb(III), to the two catalytic cysteine residues (Cys52(SG)–Ag = 2.0 Å; Cys57(SG)–Ag = 2.3 Å) and to the His461' of the 2-fold symmetry related subunit (His461'(ND1)–Ag = 2.7 Å) (Figure 2). The fourth coordination ligand is Thr335 (Thr335(OG1)–Ag = 3.3 Å). Thus, the structural analysis clearly shows that silver binds in the catalytic cleft as Sb(III) and inhibits TR activity by coordinating the same residues. Similarly to what observed for Sb(III), silver binding is only possible upon enzyme reduction, because in the oxidized enzyme the two cysteine residues form a disulfide bridge.

Interestingly, inspection of the structure allowed us to identify a new dinuclear metal binding site placed at the interface between the 2-fold symmetry related subunits. The silver atoms are placed at a distance of 3.3 Å from each other, bound in a cavity formed by the two 433–437 α -helices of both subunits and coordinated by residues furnished by both subunits. The first Ag(0), displaying an occupancy of 100%, is linearly coordinated by the Cys444 residues of both subunits (S–Ag distances: 1.8 Å and 1.9 Å). The second Ag(0), displaying an occupancy of 50%, is linearly coordinated by Met447 and Met447' (S–Ag distances: 2.2 Å and 2.4 Å) (Figure 3). It cannot be excluded that these two silver ions may contribute to enzyme inhibition by increasing the rigidity of the protein scaffold.

To test the silver antiparasitic activity on *Leishmania* cells in both amastigote and promastigote stages, we used AgNPs as silver-delivery agents (for experimental details, see Supporting Information). Nanosized particles have elicited much attention in recent years for their chemical and physical properties with respect to ionic and bulk material. In fact, recent studies demonstrated that AgNPs have a strong microbicidal effect and that this effect depends on the surface area as well as the shape of the nanoparticles.¹⁶ In addition, the use of the cage-like protein PffT as bioreactor allows AgNP to be produced with a narrow size distribution and high stability in water solution at millimolar particle concentrations. Conversely, other AgNPs with adequate solution stability could be obtained only at submicromolar levels.¹³

Further, PffT is a suitable carrier for antileishmanial drugs, since ferritins are known to be phagocitized by macrophages, where the amastigotes proliferate, thereby guaranteeing a high concentration of the encapsulated cargo inside the target cell.¹⁵

The AgNPs induced a potent dose-dependent antiproliferative effect on *L. infantum* promastigotes, the extracellular *Leishmania* stage. A dose of 50 μ M Ag induced 100% promastigote mortality, whereas concentrations ranging from 1 to 10 μ M determined a leishmanistatic activity. At these sublethal concentrations, parasites showed motility inhibition and body swelling, not present in control cells. Lower concentrations, ≤ 0.2 μ M, did not affect either promastigote growth or morphology. The IC₅₀ deduced from the dose–response curve was 2.18 ± 0.33 μ M (Figure 4A).

The efficacy of AgNPs on the amastigote stage of *L. infantum* was evaluated in murine macrophages from Balb/c mice.¹⁷ Drug concentrations were selected according to the observed activity against promastigotes. The samples of AgNPs containing 8–10 μ M Ag(0) were found to be highly toxic for the macrophage cells, provoking cell lysis. However, the toxic effect of AgNPs on macrophages was shown to be dose-dependent and associated with a leishmanicidal activity in the concentration range 0.2–4 μ M. At these concentrations, AgNPs produced a reduction in intracellular amastigote number ranging from 10.8 to 65.0%. The AgNPs IC₅₀, deduced from the dose–response curve, was 1.76 ± 0.24 μ M (Figure 4B). At concentrations ≤ 0.1 μ M, the PffT–AgNPs complex exhibited neither macrophage toxicity nor efficacy against the parasites.

The IC₅₀ values measured for both promastigote and amastigote stages are lower than the IC₅₀ of antimonial drugs in free and encapsulated forms, which ranges from 30 to 900 μ M.¹⁸ AgNPs showed leishmanicidal activity on the amastigote stage, in the concentration range 0.2–4 μ M. However, a dose-dependent cytotoxicity phenomenon toward the host cells was observed at concentrations ≥ 4 μ M. A safe range of AgNPs concentrations for the treatment of *Leishmania* infections can only be defined after appropriate *in vivo* studies.

In conclusion, the biochemical analysis here reported on reduced TR from *L. infantum* in complex with silver shows that this metal can inhibit TR at nanomolar concentrations and, therefore, is more effective than antimony *in vitro*. The structural analysis clearly demonstrates that the mechanism of inhibition is very similar to that of Sb(III). Upon TR reduction by NADPH, silver binds to the catalytic triad (Cys52, Cys57, and His461') of the enzyme, thereby disallowing the hydride transfer from the protein to the trypanothione. These results are confirmed

by *in vitro* experiments on both promastigote and amastigote stages, which showed that the PfFT-AgNPs are able to kill the parasite on both stages at micromolar concentrations. Although silver, as other metals (including antimony), binds to the thiols of many proteins, and selectivity issues could be raised, the authors strongly believe that the use of a novel approach, based on protein-encapsulated metals, may represent a promising strategy for the treatment of cutaneous leishmaniasis.

■ ASSOCIATED CONTENT

S Supporting Information. Information on structure solution and overall fold, experimental procedures for protein crystallization and structure solution, TR inhibition assays, preparation of Ag(0) nanoparticles, promastigote and amastigote-macrophage assays, a figure showing the mechanism of trypanothione reduction by TR (Figure S1), and a figure showing experimental traces, Lineweaver–Burk plots, and Dixon plots (Figure S2). This information is available free of charge via the Internet at <http://pubs.acs.org>.

Accession Codes

Atomic coordinates and structure factors have been deposited in the Protein Data Bank (www.ebi.ac.uk/msd) with PDB accession code 2X50.

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Author Contributions

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■ ABBREVIATION LIST

TR, trypanothione reductase; AgNPs, silver nanoparticles; PfFT, *Pyrococcus furiosus* ferritin; T(SH)₂, reduced trypanothione; TryS, trypanothione synthetase; TrxR, thioredoxin reductase; NPs, nanoparticles; NADPH, reduced nicotinamide adenine dinucleotide phosphate; FAD, flavin adenine dinucleotide; TS₂, oxidized trypanothione; Tris, tris(hydroxymethyl)aminomethane/HCl; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

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