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ESI MS Studies of *T. Brucei* Trypanothione Reductase and its Reactions With Selected Metal Compounds

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Trypanothione reductase from *Trypanosoma brucei* (*Tb*TR) is a key enzyme of the parasite responsible for human African trypanosomiasis. *Tb*TR catalyzes the reduction of trypanothione, an antioxidant dithiol that protects trypanosomatid parasites from oxidative stress induced by the mammalian host as a defense system. *Tb*TR is considered an attractive target for the development of novel anti-parasitic drugs because it is essential for parasite survival and has no close homologues in humans. Previous studies have shown that metal compounds can act as potent inhibitors of trypanothione reductase and of related enzymes and may be considered for disease treatment. To better understand the molecular basis of such inhibition, we report the study of the interactions of a small group of metal compounds with *Tb*TR, analyzed by ESI MS measurements. This technique allows direct visualization of the enzyme in solution and the detection and characterization of the metal binding process. Accordingly, *Tb*TR was reacted with a panel of metal compounds [Q1] [Q2] including thimerosal, auranofin, its halogen analogues, and bismuth acetate. ESI MS results provide a detailed description of the interactions taking place for each metal compound tested. The implications of these results for the development of novel metal-based compounds [Q3] [Q4] as potential anti-trypanosomal agents are discussed.

Introduction

Human African trypanosomiasis (HAT), also known as sleeping sickness, is a serious health threat in sub-Saharan Africa, where more than 70 million people are at risk of infection.[1] Caused by the protozoan parasites *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*, HAT spreads from the bloodstream to the central nervous system causing severe neurological symptoms.[2] Without treatment, the disease is usually fatal.[3] Current drug treatments for HAT are inadequate due to poor efficacy, significant side effects and to the necessity of parenteral administration. These drawbacks highlight the urgent need to find novel and more effective anti-trypanosomal drugs.

A key feature shared by all trypanosomatids is that their defense mechanism against host-induced oxidative stress relies on the trypanothione disulfide system (Figure 1), an unusual variant of glutathione unique to these organisms.[4,5] Reduced trypanothione is involved in several fundamental cellular processes, including redox homeostasis, metals and drugs detoxification, deoxyribonucleotide synthesis and suppression of apoptosis. Enzymes involved in this pathway are therefore considered interesting candidates for the development of new drug treatments.

Among these, the NADPH-dependent flavoprotein disulfide oxidoreductase trypanothione reductase, the enzyme directly

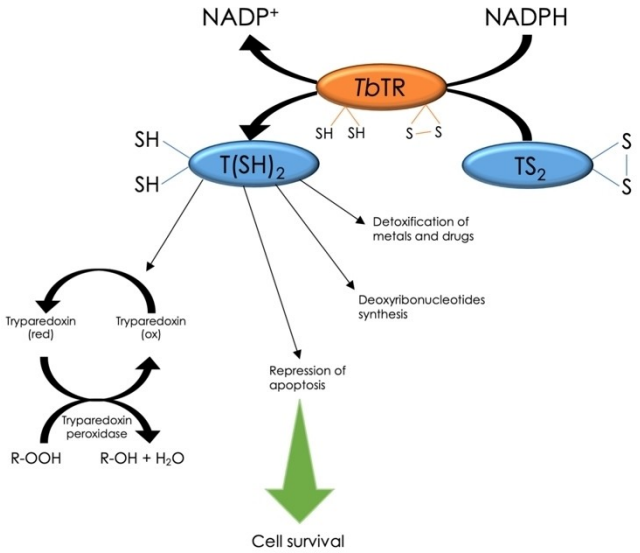

Figure 1. The trypanothione disulfide system. Trypanothione is directly maintained in the reduced state at the expense of NADPH by trypanothione reductase. In turn, trypanothione reduces various substrates, including tryparedoxin, and is thus involved in several fundamental processes for parasite survival.

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responsible for maintaining trypanothione in the reduced state, has been extensively studied because it is:

(i) essential for parasite survival;
(ii) absent in the host, where TR is replaced by glutathione reductase;
(iii) druggable, as it can be effectively targeted by inhibitors;
(iv) highly homologous in sequence and structure among trypanosomatids, making it a valuable target for the development of a single broad-spectrum drug.

The X-ray crystal structure of *Trypanosoma brucei* trypanothione reductase (*Tb*TR) has been solved at high resolution (2.1–1.98 Å resolution), revealing that the enzyme forms homodimers in solution.[6,7] Each monomer weighs around 53 kDa and is characterized by the presence of a FAD-binding domain, a NADPH-binding domain and an interface domain (Figure 2).

Some studies have reported on the interactions of inorganic compounds with these enzymes.[6–10] Notably, a few metal-based/inorganic drugs, such as the organoarsenical melarsoprol, are currently used in the treatment of trypanosomiasis. Previous studies on the closely related enzyme from *Leishmania infantum* have shown that this enzyme can be effectively inhibited by antimony and gold-based compounds.[11–13] In particular, the latter metals can coordinate the two redox-active catalytic cysteine residues (Cys52 and Cys57), which are responsible for the binding and release of trypanothione and are conserved within a large variety of trypanothione reductases including the one of *Trypanosoma brucei*.[12,13]

Electrospray ionization mass spectrometry (ESI MS) has proven to be an excellent method to characterize the behavior of several proteins in solution and to study their interactions with metallodrugs.[14–18] Indeed, thanks to recent technological advances, ESI MS offers the possibility to monitor *in real time* the solution behavior of proteins/enzymes as large as *Tb*TR (MW 106 kDa) and to characterize their interactions with a variety of metal species that could act as enzyme inhibitors.

Based on the above arguments we decided to explore through ESI MS the solution behavior of TbTR and the adducts that the enzyme may form upon reaction with a small panel of metal compounds of medicinal interest.

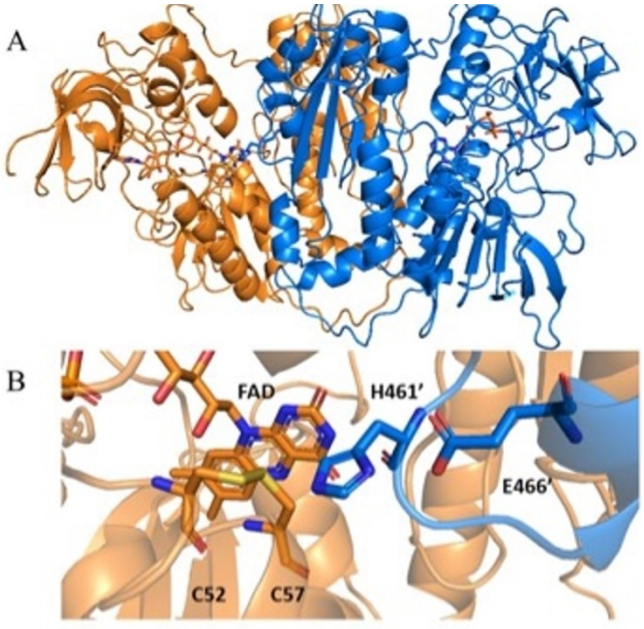

Figure 2. A) The X-ray diffraction structure of the enzyme Trypanothione Reductase from *Trypanosoma brucei* (PDB entry 2WOI).[8] The two monomers are in slate blue and orange. B) Blow-up of the catalytic site. The coenzyme FAD and the residues important for catalysis are shown as sticks and labelled in the figure. The atoms of the residues are colored as follows: the carbons of chain A in orange and those of chain B in slate blue, oxygens in red, nitrogens in blue, and sulfurs in yellow.

Results and Discussion

ESI-MS Measurements of the Native Enzyme

The native enzyme, prepared as described in Materials and Methods, was subjected to ESI MS analysis in the direct infusion mode. To evaluate the protein's stability toward temperature, different samples dissolved in 20 mM ammonium acetate pH 6.8 with 0.5 % v/v HEPES were incubated at 4 °C, 22 °C (room temperature) and 37 °C, with the first condition resulting to be the [Q5] [Q6] ideal one. The protein was then incubated with an excess of the reducing agent dithiothreitol (DTT) to reduce the active site cysteines, which are known to form a disulfide bond, and an ESI MS spectrum was acquired after 30 minutes of incubation at 4 °C to assess the effect of DTT treatment on the protein. As shown in Figure 3, DTT had no effect on perturbing the stability of the protein. We found that the protein remained stable for up to 48 hours at 4 °C under these experimental conditions.

Remarkably, in both cases the deconvoluted ESI MS spectrum is very well resolved and shows a dominant peak located at 53438 daltons, which agrees well with the expected molecular weight of the amino acid sequence of the *Tb*TR monomer without the FAD molecule. A peak of much lower intensity, the nature of which has not been determined yet, is also detected at 53620 daltons.

The Panel of Metal Compounds

For this study we have prepared a small panel of metal compounds that includes: the gold(I) based drug auranofin, its halogen analogues chloro(triethylphosphine) gold(I) and iodo(triethylphosphine)gold(I), the vaccine adjuvant sodium thiosalicylate ethylmercury (*i.e.*, thiomersal, or thimerosal) and bismuth acetate. These metal compounds were chosen because, according to HSAB theory, their metal centers are "soft Lewis acids" with an expected high binding affinity to soft bases such as the sulfur atom of a cysteine residue, and because they are medicinally relevant and have already been used in pharmaceuticals. The chemical structures of the panel compounds are shown in Figure 4.

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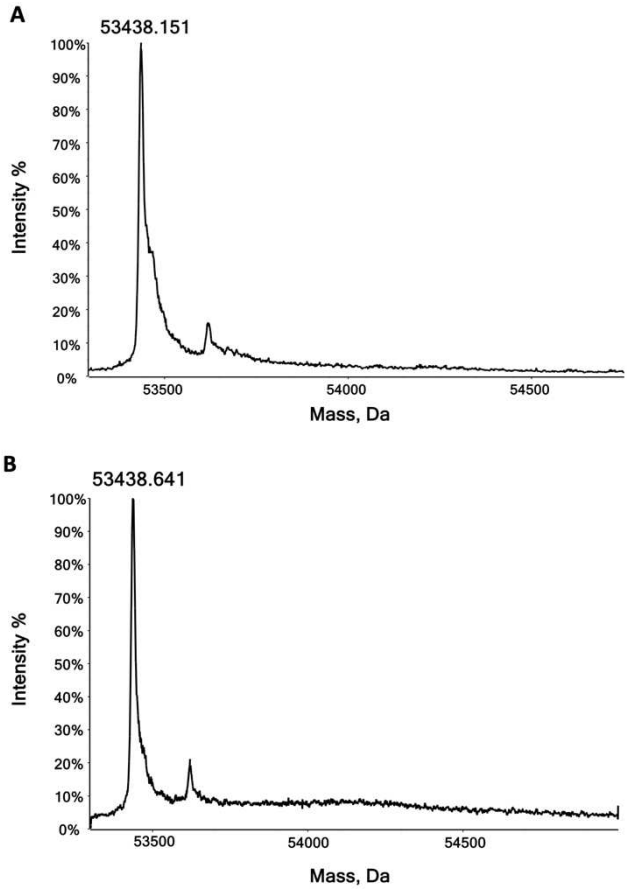

Figure 3. ESI MS spectrum recorded at 4 °C after 24 hours of: A) the native enzyme; B) the native enzyme treated in a 1 : 14 protein monomer-to-DTT ratio.

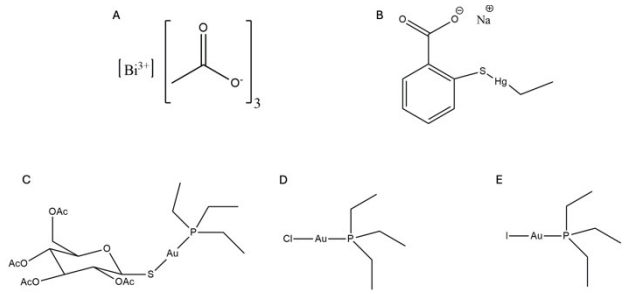

Figure 4. The investigated metal compounds: A) bismuth(III) acetate; B) sodium thiosalicylate ethylmercury (*i.e.* thimerosal); C) auranofin; D) chloro(triethylphosphine)gold(I); E) iodo(triethylphosphine)gold(I).

Reactions of *Tb*TR with Panel Compounds

The enzyme was treated with the above panel compounds containing soft metal centers (Figure 4), which typically have a high affinity for free thiol groups of proteins. Notably, treatment of *Tb*TR with these metal compounds for 24 hours resulted in a partial conversion of the native enzyme into the corresponding metalated adduct. The resulting ESI MS spectra documenting adduct formation are described below.

First, *Tb*TR was treated with auranofin and its chloride and iodide analogues at a 1 : 3 protein monomer-to-metal molar ratio. The spectra, recorded after 24 hours of incubation, are shown in Figure 5. In all cases the resulting ESI MS spectra well document the formation of a typical 1 : 1 adduct between the protein and an $AuPEt_3$ (gold triethylphosphine) fragment. Under the applied experimental conditions, the intensity of the peak of the 1 : 1 adduct is significantly lower than that of the unmodified native protein, ranging from 70 % to 50 %, indicating that only partial protein metalation has occurred. Some additional peaks of far lower intensity are seen at larger mass values, which are tentatively assigned to adducts with a protein-to-metal stoichiometry of 1 : 2.

*Tb*TR was then reacted with the mercury compound thimerosal. The ESI MS spectrum of *Tb*TR treated with thimerosal in a 1 : 3 protein monomer-to-metal molar ratio for 24 hours is shown in Figure 6. From the obtained mass spectrum, it is evident that treatment with thimerosal results in the formation of a small amount (approximately 20 % in terms of relative peak intensity) of a well-defined adduct bearing an

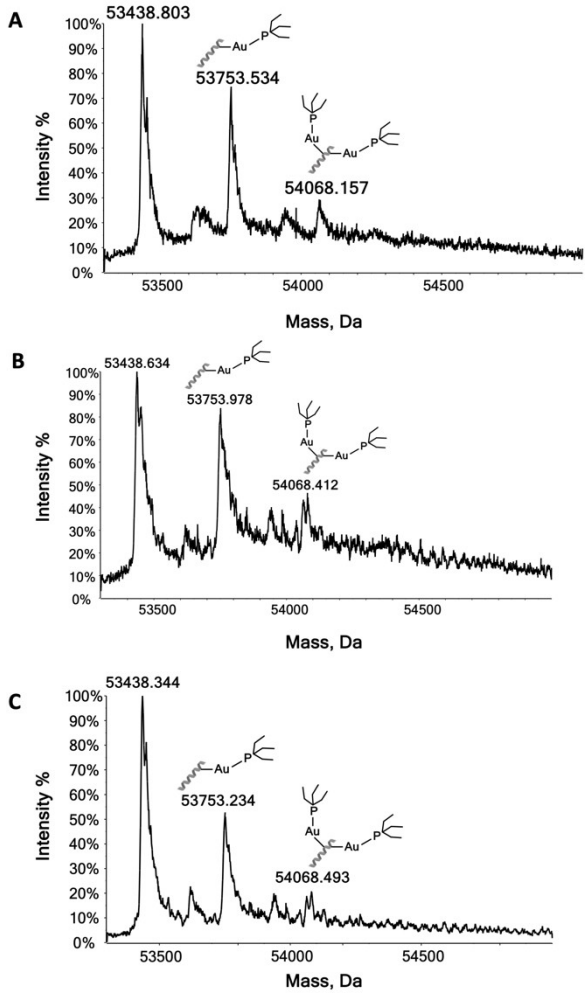

Figure 5. ESI MS spectrum recorded at 4 °C after 24 hours of incubation of the enzyme in a 1 : 14 : 3 protein monomer-to-DTT-to-metal ratio of: A) auranofin; B) chloro(triethylphosphine)gold(I); C) iodo(triethylphosphine)gold(I).

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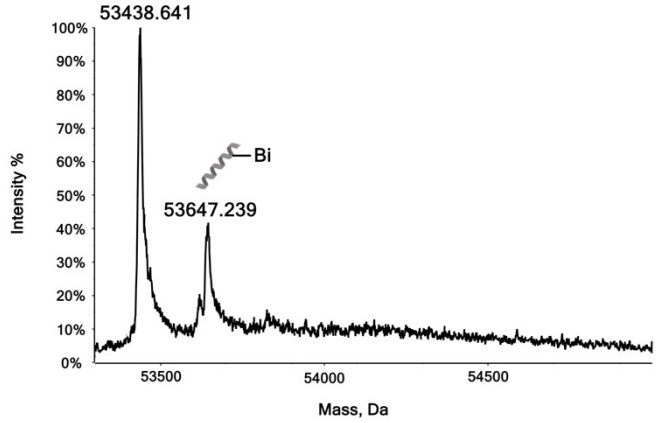

Figure 6. ESI MS spectrum recorded at 4 °C after 24 hours of incubation of the enzyme in a 1:14:3 protein monomer-to-DTT-to-thimerosal molar ratio.

ethylmercury fragment, as documented by the appearance of a new peak at 53667 Da. This suggests that thimerosal preferentially releases the thiosalicylate ligand upon interaction with the protein.

Finally, *Tb*TR was reacted with bismuth(III) acetate. The ESI MS spectrum of *Tb*TR treated with bismuth acetate, in a 1:3 protein monomer-to-metal molar ratio for 24 hours is shown in Figure 7. It is evident that treatment with this compound results in the formation of a well-defined adduct carrying a single bismuth ion, as documented by the appearance of a new peak at 53647 Da, beyond the peak of the native enzyme and its satellite.

To gain more insight into the rate of the metalation process and the stability of the resulting adducts, additional ESI MS spectra were obtained for all the above samples after 3 hours of incubation. The 3 hours incubation spectra are reported in SI (Figure S1). Notably, when these latter spectra are compared with those recorded after 24 hours, we observe that the formation of the adducts is almost complete after just 3 hours of incubation. This suggests that the process of protein metalation is relatively fast for all panel compounds and takes only a few hours.

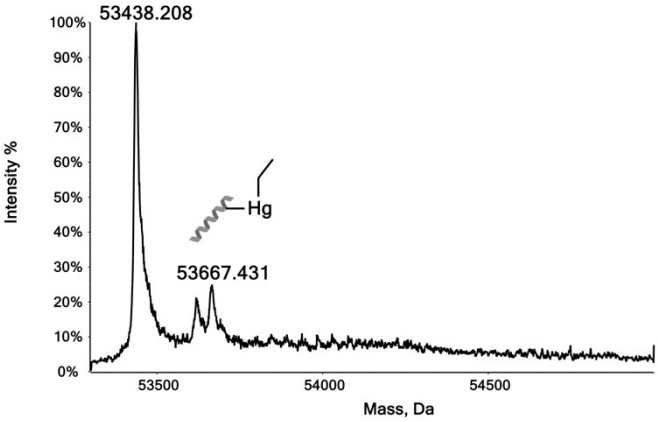

Figure 7. ESI MS spectrum recorded at 4 °C after 24 hours of incubation of the enzyme in a 1:14:3 protein monomer-to-DTT-to-bismuth acetate molar ratio.

Previous studies suggest that the two conserved cysteines located in the active site of *Tb*TR, i.e. Cys52 and Cys57, are the most likely anchoring points for the above metal species. In the native protein, these two cysteines participate in the formation of a disulfide bridge that is broken by pretreatment with DTT to obtain the functional form of the enzyme. This led us to perform an additional experiment where *Tb*TR was reacted with auranofin in a 1:1 protein monomer-to-metal molar ratio, either in presence or in absence of DTT. Notably, adduct formation was only observed after DTT treatment (Figure S2). This result supports the idea that active site cysteines are indeed the primary binding site for auranofin in *Tb*TR.

Conclusions

*Tb*TR is an important enzyme that represents a primary target for the development of new antitrypanosomal drugs. Previous studies have shown that this type of enzyme can be effectively inhibited by various metal compounds, including antimony and gold compounds. The inhibition is most likely due to coordination of these metal species to the two conserved active site cysteines that are catalytically relevant.

We have shown here that ESI-MS, after careful adjustment of the solution and instrumental conditions, is an excellent biophysical tool to monitor the solution behavior of *Tb*TR and to characterize its interactions with metallic species. Indeed, we were able to obtain well-resolved spectra documenting adduct formation when *Tb*TR was challenged with a small panel of medicinal metal compounds, including three gold(I) compounds, the mercury compound thimerosal and bismuth(III) acetate.

From the analysis of the ESI MS spectra concerning the interactions of *Tb*TR with the above metal compounds, the following conclusions can be drawn:

1. All metal compounds tested react with the native enzyme to form the corresponding 1:1 adduct.
2. In all cases the protein metalation reaction is relatively fast and almost complete after 3 hours of incubation at 4 °C.
3. The amount of adduct formed is relatively small, as the intensity of the corresponding ESI MS peak is only 20–70% of the intensity of the peak of the native protein.
4. The nature of the metal fragments bound to the enzyme is unambiguously determined from the observed mass shifts.

Based on these results, we can say that:

- The enzyme trypanothione reductase can be successfully monitored in solution in real time by ESI MS measurements.
- The ESI MS spectra are well resolved and characterized by an intense peak corresponding to the monomeric species.
- Samples are stable for hours under standard solution conditions.
- Treatment with a variety of metal compounds results in the formation of the corresponding metalated species, which carry characteristic metal fragments.

Interestingly, in the case of auranofin, a preferential gold binding to active site cysteines, that is expected to result in potent enzyme inhibition, was documented.[12] This finding provides a solid molecular basis for the role of metal compounds as potential antitrypanosomal drugs. Attempts will be made to correlate the information arising from the ESI MS studies of the metallodrug- protein adducts with their enzyme inhibition potencies.

Based on these considerations, we can predict that the extensive use of ESI MS analysis, complemented by enzyme inhibition assays, may allow the identification of novel and better metal compounds for the treatment of trypanosomiasis. Special attention will be given to the possibility of repurposing clinically established or clinically approved metal-based drugs as potential antitrypanosomal agents, as their implementation in patient treatment practice could be far more straightforward.

Experimental Section

***Tb*TR cloning, expression and purification**. The production of TbTR enzyme was performed as previously described, with slight changes.[19] Briefly, the gene coding for the enzyme (aa 1–492) was obtained from GenScript and codon optimized for the expression in E. coli. The sequence was cloned in pET15b vector using the restriction site NdeI and XhoI, to maintain an N-terminal 6xHistag for purification purpose. The construct TbTR_pET15b was transformed in E. coli BL21 (DE3) cells. TbTR was overexpressed in LB medium upon addition of 1 mM IPTG when the O.D. at 600 nm reached 0.6. E. coli cultures were incubated 4 more hours at 37 °C prior cell harvest by centrifugation (6000 RPM, 30 min at 4 °C). Cell pellets were then re-suspended in lysis buffer (50 mM Tris pH 8.0, 0.3 M NaCl, 5 mM imidazole, 5 mM MgCl, 2 0.1 mM PMSF, 1 Pierce protease inhibitor cocktail tablet), incubated at 4 °C for 30 min with strong agitation and lysed by sonication. The soluble fraction was collected by centrifugation (11000 RPM, 30 min at 4 °C) and then loaded onto 5 ml of HiTrap Ni-NTA resin (Cytiva). TbTR was eluted using a gradient of imidazole. Finally, the buffer was exchanged into 20 mM HEPES pH 7.4 and TbTR was frozen in liquid nitrogen and stored at −80 °C in small aliquots. The purity of recombinant TbTR was evaluated by SDS PAGE gel chromatography.

Electrospray ionization mass spectrometry materials and experimental conditions. Auranofin (purity > 98 %) was purchased from AdipoGen Life Sciences. Starting from the commercially available auranofin analogue chloro(triethylphosphine)gold(I) (Sigma–Aldrich) in which the thiosugar ligand is replaced by one chloride ligand, an iodide auranofin analogue -iodo(triethylphosphine)gold(I)- was synthesized.[20] Thimerosal and dithiothreitol (DTT) were purchased from Fluka. Bismuth(III) acetate was purchased from Thermo Fisher Scientific. LC–MS grade water was purchased from Merck. Ammonium acetate was purchased from Sigma-Aldrich.

Stock solutions 10^{-2} M of the gold(I) compounds were prepared by dissolving the samples in DMSO. Stock solutions 10^{-2} M of thimerosal and bismuth(III) acetate were prepared by dissolving the samples respectively in LC–MS grade water and LC–MS grade water with 10 % v/v of acetic acid. Aliquots of *Tb*TR stock solutions were mixed with the reducing agent dithiothreitol at a protein monomer-to-DTT ratio of 1 : 14 for 30 min at 4 °C. Subsequently, aliquots of each metal compounds were added at a protein monomer-to-metal ratio of 1 : 3 and diluted with 20 mM ammonium acetate to a 10^{-4} M final protein concentration. The mixtures were incubated at 4 °C up to 24 h.

After the incubation time, all solutions were sampled and diluted to a final protein concentration of 5×10^{-6} M using 20 mM ammonium acetate. The final solutions were also added with 0.5 % v/v of formic acid just before the infusion in the mass spectrometer.

The ESI mass study was performed using a TripleTOF® 5600+ high-resolution mass spectrometer (AB Sciex, Framingham, MA, United States), equipped with a DuoSpray® interface operating with an ESI probe. Respective ESI mass spectra were acquired through direct infusion at 7 μL/min of flow rate. The general ESI source parameters optimized for *Tb*TR analysis were as follows: positive polarity, ion spray voltage floating 5500 V, temperature 0° C, ion source Gas 1 (GS1) 40 L/min; ion source Gas 2 (GS2) 0; curtain gas (CUR) 20 L/min, collision energy (CE) 10 V; declustering potential (DP) 200 V, acquisition range 900–3000 m/z. For acquisition, Analyst TF software 1.7.1 (Sciex) was used, and deconvoluted spectra were obtained by using the Bio Tool Kit micro-application v.2.2 embedded in PeakViewTM software v.2.2 (Sciex).

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: ESI mass spectrometry · Metalated protein · Metal-based drugs · Parasitic infection

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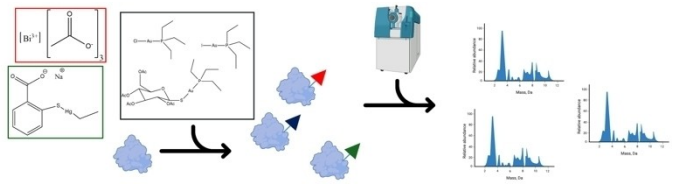

The study investigates the interactions between Trypanothione reductase from *Trypanosoma brucei* (*Tb*TR) and selected metal compounds using ESI-MS measurements. The findings describe how these compounds bind to *Tb*TR, highlighting their potential as novel anti-parasitic agents by targeting this crucial enzyme, which is essential for the parasite's survival.

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ESI MS Studies of *T. Brucei* Trypanothione Reductase and its Reactions With Selected Metal Compounds

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