Chapter 30
Redox-Active Metal Complexes in Trypanosomatids

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Abbreviations

ABC	ATP-binding cassette
Cys	Cysteine
Cys-Gly	Cysteinyl-glycine
GSH	Glutathione
iNOS	Inducible nitric oxide synthase
NO	Nitric oxide
MRPA	Multidrug resistance-associated protein A
ROS	Reactive oxygen species
TR	Trypanothione reductase
$T(SH)_2$	Reduced trypanothione
WHO	World Health Organization

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30.1 Historical Use of Metal Complexes Against Trypanosomatids

Trypanosomatids are protozoan parasites that cause various diseases in human, such as leishmaniasis, Chagas disease, and sleeping sickness. According to World Health Organization (WHO), trypanosomiasis and leishmaniasis are the most challenging among the neglected tropical diseases [1, 2].

At the beginning of the last century, Paul Ehrlich, considered the father of modern chemotherapy, discovered the anti-trypanosomal arsenic-based drug, atoxyl, for treatment of sleeping sickness [3]. However, atoxyl caused side effects, mainly blindness. In 1934, Ernst Friedheim, in the search for a safer drug, designed and developed melarsoprol which saved three millions of lives in the 1940s [4]. This arsenical drug is still in use for sleeping sickness, but is limited to cases of advanced infections with *Trypanosoma brucei gambiense* and *rhodesiense*. More recently, trivalent arsenicals in the form of As_2O_3 started to be used clinically in the treatment of acute promyelocytic leukemia [5].

Antimony-based drugs have also a long history of use in the treatment of infectious diseases. The importance of antimony in the early medicine is well-documented due to the debate created around their utilization in this period [6]. The first antimony compounds prepared for medicinal applications were introduced in the sixteenth and seventeenth centuries, with emphasis on antimony(III) potassium tartrate (tartar emetic). Tartar emetic was first obtained placing sour wine in glasses of antimony metal and its use was prescribed for many diseases, especially the lung diseases. During the late nineteenth century, tartar emetic was used for fever and pneumonia and its use declined slowly until the beginning of twentieth century.

At the beginning of the last century, the Brazilian physician Gaspar Vianna, pioneer researcher in the treatment of leishmaniasis, reported the efficacy of tartar emetic for clinical treatment of muco-cutaneous leishmaniasis [7]. In India, Brahmachari discovered in 1912 the organoantimonial compound urea stibamine, the first effective drug against visceral leishmaniasis, and was a nominee for the Nobel Prize in 1929 for this finding [8].

From the 1940s, these antimonials were substituted by the less toxic pentavalent antimony (Sb(V)) complexes, meglumine antimoniate and sodium stibogluconate (Fig. 30.1), in the therapeutics of leishmaniases. Typically, these Sb(V) drugs are given at 20 mg of Sb/kg per day intramuscularly or intravenously for 20–30 days [9]. Even though pentavalent antimonials are still the first-line drugs against all forms of leishmaniasis in most developing countries, their mechanism of action is still not fully understood and their use in the clinical setting is limited by their side effects and the emergence of resistance to antimony [9].

Recent advances in understanding the molecular and cellular biology of leishmaniasis as well as the cellular accumulation pathways, mechanisms of resistance, and target identification allow a more systematic rationale-based approach for development of new anti-parasitic drugs [10–12]. In this context, complexes with other metals including gold showed promising pharmacological activities.

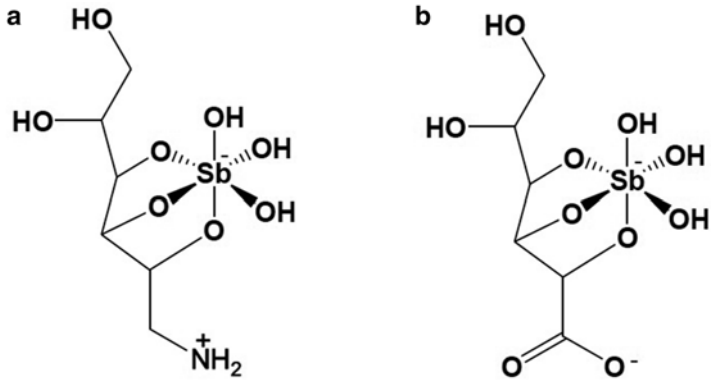

Fig. 30.1 Structural formula proposed for meglumine antimoniate (**a**) and stibogluconate (**b**). Adapted from Frézard et al. [9]

Most of the progress regarding drug development and mechanism of action was achieved using cell and animal models of leishmaniasis [13]. The in vitro models include extracellular log-phase leishmania promastigotes and established axenic leishmania amastigotes and the intracellular amastigotes in primary mouse peritoneal macrophages or monocyte transformed macrophages as host cells. The in vivo models use mostly inbred strains of mice infected with leishmania species causing either cutaneous or visceral leishmaniasis.

Interestingly, in the case of these metal-based compounds, evidence was obtained that those exert their antiparasitic action by causing oxidative stress. This chapter will describe in details the current knowledge on their mechanism of action, with emphasis on antimonial drugs and gold complexes for leishmaniasis.

30.2 Importance of Redox Systems in the Host–Leishmania Parasite Relationship

The protozoan leishmania parasite has a relatively simple life cycle with two principal stages: the flagellated mobile promastigote living in the gut of the sandfly vector and the intracellular amastigote within phagolysosomal vesicles of the vertebrate host macrophage. After recognition of *Leishmania* spp., macrophages are activated, triggering phagocytosis and various cellular processes to destroy the parasite. These cellular processes include production of phagolysosomal degradation enzymes, oxidative burst generation, and nitric oxide (NO) production. The oxidative burst provoked by the enzyme NADPH oxidase is a result of the increase in oxygen consumption as a consequence of the phagocytosis process. After macrophage activation, increased concentrations of various cytokines such as IFN-γ and TNF-α enhance NADPH oxidase activity and subsequently production of reactive oxygen species (ROS), such as superoxide radical. The production of superoxide

radical leads to the spontaneous or enzymatic formation of hydrogen peroxide, hydroxyl radical, hypochlorite, and peroxynitrite. The increased NO and NO-metabolite levels in activated macrophages are the result of inducible nitric oxide synthase (iNOS) activation. Parasite persistence within the macrophages is determined by a balance between the ability of the immune response to sufficiently activate *Leishmania*-infected macrophages and the ability of the parasite to resist cytotoxic mechanisms of macrophage activation [14].

Although *Leishmania* species are susceptible in vitro to exogenous superoxide radical, hydrogen peroxide, nitric oxide, and peroxynitrite, they manage to survive the endogenous oxidative burst during phagocytosis and the subsequent elevated nitric oxide production in the macrophage. The parasite adopts various defense mechanisms against oxidative stress: the lipophosphoglycan membrane decreases superoxide radical production by inhibiting NADPH oxidase assembly and the parasite also protects itself through antioxidant enzymes [15]. Among the various parasite defense mechanisms against host attack, thiol metabolism appears as a first-line defense. One major system involved in the redox homeostasis in trypanosomatids is the trypanothione ($T(SH)_2$)/trypanothione reductase (TR)system. The ($T(SH)_2$)/TR system, which keeps $T(SH)_2$ under the reduced state, replaces the nearly ubiquitous glutathione/GR system, protects trypanosomatids from oxidative damage, and delivers the reducing equivalents for DNA synthesis [16].

It has been reported that $T(SH)_2$ is capable of reducing NO (generated by the host cell) and iron into harmless stable dinitrosyl iron complex with 600 time greater affinity than mammalian glutathione (GSH) reductase system [17]. This is the mechanism by which the parasite protects itself from such lethal environment.

30.3 Pentavalent Antimonial Drugs Against Leishmaniasis

The metabolism and mechanism of action of pentavalent antimonials against leishmania parasites are not fully understood [9]. However, the data available so far converge towards the central role of redox processes.

30.3.1 Drug Activation Though Metal Reduction

It is generally assumed that Sb(V) behaves as a prodrug, undergoing reduction within the organism into the more toxic and active trivalent form. This model is supported by several reports that Sb(V) is reduced into Sb(III) in the vertebrate host and that reduction could also happen in the parasite [18–22]. However, the exact microenvironment where the reduction occurs is still unclear.

Leishmania promastigote forms are insensitive to Sb(V). On the other hand, amastigote-like cultured parasites are sensitive to pentavalent antimonials, suggesting the occurrence of intracellular Sb(V) reduction in this life-cycle stage [23].

However, in the latter case, thiol availability from the culture medium and low pH as well as high temperature may also be determinant factors in Sb(V) to Sb(III) reduction and consequently high sensitivity to Sb(V) [24, 25].

The higher sensitivity of intramacrophagic amastigotes to Sb(V) compared to axenic amastigote-like favors the hypothesis of host-associated Sb(V) to Sb(III) reduction [23].

Thiols have been reported as potential reducing agents in this conversion [24, 26]. Reduction of Sb(V) to Sb(III) was found to occur spontaneously in the presence of the following thiols: (1) GSH, which is the main thiol in the cytosol of mammalian cells; (2) cysteine (Cys) and cysteinyl-glycine (Cys-Gly) found predominantly within lysosomes; and (3) the bis(glutathione)-spermidine conjugate $T(SH)_2$, which is the main thiol within the parasite [27].

The observations that Cys, Cys-Gly, and $T(SH)_2$ are more effective reducing agents than GSH and that this reaction is favored in acidic pH [18] led to the hypothesis that Sb(V) may be reduced in vivo by $T(SH)_2$ within leishmania parasites and by Cys or Cys-Gly within the acidic compartments of mammalian cells. Both promastigotes and intracellular amastigotes maintain intracellular pH values close to neutral (~7) even in the presence of extracellular acidic pH (~4–5). This observation reinforces the hypothesis that thiol-mediated Sb(V) to Sb(III) reduction takes place preferentially in the host cell.

Other studies have suggested the participation of a parasite-specific enzymes in the process of reduction of Sb(V) to Sb(III) [28, 29]. However, these parasite-specific enzymes are expressed in both life-cycle stages and therefore it is difficult to accommodate the different toxicity of pentavalent antimony toward promastigotes and amastigotes.

Recently, the pentavalent antimonial drug meglumine antimoniate was found to contain up to 30 % of Sb(III), indicating that the mode of action of this drug could be mediated by this residual amount of Sb(III) [30]. Additionally, the availability of Sb(III) increased at low pH values, suggesting that this drug may act as molecular carrier releasing the active $Sb(OH)_3$ form specifically in the acidic intracellular compartment where leishmania parasite resides.

30.3.2 Antimony-Induced Redox Imbalance

Sb(III) is classified as a borderline metal ion within Pearson's hard-soft acid-base theory and has a high affinity towards nitrogen- and sulfhydryl-containing ligands. The anti-leishmanial mechanisms of Sb(III) are probably related to its interaction with sulfhydryl-containing biomolecules, including thiols, peptides, proteins, and enzymes.

Thus, Sb(III) was found to form stable complex with the major intracellular thiols, GSH and $T(SH)_2$, in the form of a 1:3 and 1:1 Sb-thiol complexes, respectively [24, 26, 31]. Once Sb(III) is in the cell and conjugated to $T(SH)_2$, the complex can be sequestered inside a vacuole or extruded by ATP-binding cassette (ABC) transporters [32, 33].

It has been reported that Sb(III) interferes with the thiol metabolism, not only by inducing efflux of intracellular $T(SH)_2$ and GSH from intact leishmania cells, but also by inhibiting TR through specific interaction with the redox-active catalytic site [34], resulting in lower $[T(SH)_2]/[[T(S)_2]$ ratio [35]. Both actions synergistically contribute to lowering the parasite-neutralizing capacity of reactive species coming from the host. It is worth noting that similar effects in thiol metabolism were observed with Sb(V) on axenic amastigotes in these studies. Now, it is also clearly established that Sb(III) triggered apoptotic cell death associated with ROS [36].

Whether the oxidative stress resulting from metal interference in thiol metabolism is sufficient to promote cell death or additional event involving metal interaction with other molecular target(s) takes place still has to be determined. In that sense, recent studies have identified zinc-finger protein as potential target of Sb(III) [37, 38]. The zinc finger domain is characterized by the coordination of a zinc atom by several amino acid residues, including cysteine and histidine. Zinc finger proteins sharing the CCHC motif have been identified in trypanosomatids and are likely to be involved in DNA replication, structure, and repair [39]. On the other hand, CCCH zinc finger domains, which are found mainly in RNA-binding proteins with regulatory functions at all stages of mRNA metabolism [40], are suspected to play a crucial role in the biology of kinetoplastid protozoa, because of the unusual emphasis on post-transcriptional control of gene expression in this group of organisms [41]. Interestingly, Sb(III) promotes Zn(II) ejection more effectively from CCCH zinc finger peptides than CCHC peptides [38], suggesting that the action of antimonial drugs could be related mainly to interaction of Sb(III) with CCCH zinc finger proteins.

Despite the strong evidence that Sb(III) mediates the antileishmanial action of pentavalent antimonials, some studies have suggested intrinsic pharmacological actions of Sb(V) [42–44].

Demicheli and co-workers have reported the formation of a complex between adenine ribonucleoside and Sb(V). This was the first report of a physiologically relevant biomolecule capable of forming stable complexes with Sb(V). Both 1:1 and 1:2 Sb(V)-ribonucleoside complexes were evidenced [19, 42, 45–47]. The large NMR resonance changes for H2′signal suggested that –OH groups in the ribosome are the binding sites for Sb(V) probably via ring chelation at C2′ and C3′.

Stibogluconate was found to be a potent inhibitor of protein tyrosine phosphatases, which leads to an increase in cytokine responses [44]. Another recent study revealed that this drug induced generation of ROS and NO via phosphoinositide 3-kinase and mitogen-activated protein kinase activation in *Leishmania donovani*-infected macrophages [48, 49], indicating that Sb(V) can stimulate the innate arm of the immune system. Meglumine antimoniate was also reported to increase the phagocytic capacity of monocytes and neutrophils and enhance superoxide anion production by phagocytes, which represent the first line of defense against the parasite [50].

As summarized in Fig. 30.2, these data taken altogether suggest that pentavalent antimonials affect the parasite viability through both Sb(III)-induced imbalance of thiol metabolism in parasite and Sb(V)-induced stimulation of macrophage microbicidal activity.

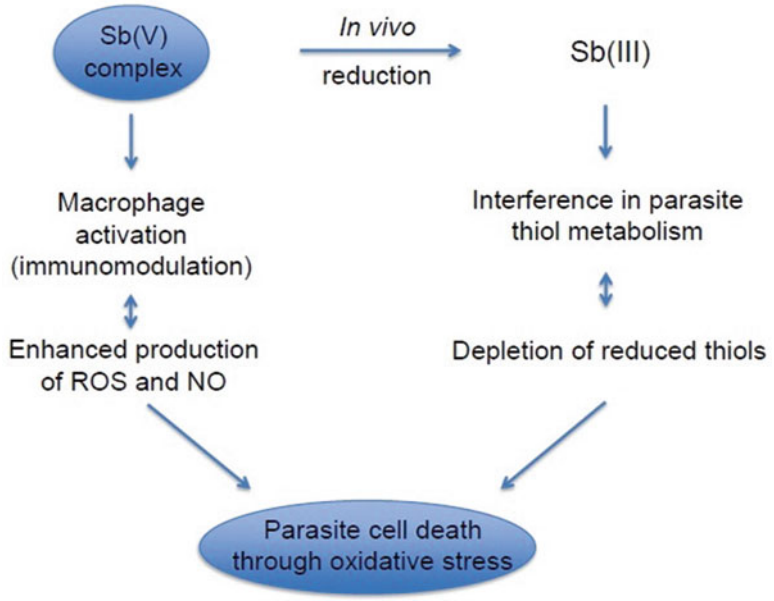

Fig. 30.2 Model for the mechanism of action of pentavalent antimonial drugs against leishmaniasis

30.3.3 Redox Changes in Antimony-Resistant Parasites

Resistance of leishmania parasites to antimony has been extensively studied in both laboratory-selected resistant leishmania lines and field isolates [51, 52, 53], allowing important insights into the molecular and functional factors that modulate parasite sensitivity to the drug.

Most antimony-resistance associated genes are involved in metabolic process related to oxidative stress, cell redox homeostasis, and thiol biosynthesis [54], which is not surprising considering the mode of action of antimonial drugs.

A change often encountered in both laboratory-selected and field-isolated resistant leishmania parasites is overexpression of rate-limiting enzymes of thiol biosynthesis, such as ornithine decarboxylase and γ-glutamylcysteine synthetase, which causes an overproduction of intracellular thiols and extra protection against the oxidative stress upon drug exposure [55–58]. The higher rate of thiol synthesis may also result in enhanced rates of efflux through ABC transporters contributing to the resistance phenotype [33]. Overexpression of the multidrug resistance-associated protein A transporter responsible for sequestration of Sb-thiol conjugates in intracellular vesicles is another frequently observed change that contributes to the resistance phenotype in *Leishmania* [32]. Thiol depletion of these strains reestab-

lished their susceptibility to Sb [57], indicating that the increased $T(SH)_2$ levels are causing resistance.

Elevated levels of tryparedoxin and tryparedoxin peroxidase, key enzymes in hydroperoxide detoxification, were also observed in antimonial resistant parasites resulting in an increased metabolism of peroxides [59].

30.3.4 Redox-Related Toxicity of Antimonial Drugs

Even though pentavalent antimonials are still the first-line drugs in several countries against all forms of leishmaniasis, their use in the clinical setting has several limitations.

Antimony therapy is often accompanied by local pain during intramuscular injection and by severe side effects that include cardiotoxicity, pancreatitis, hepatotoxicity, and nephrotoxicity [9, 60].

Although the mechanism involved in the toxicity of pentavalent antimonials is not fully elucidated, it is generally accepted that Sb(III), either present as residue in pentavalent antimonials [61] or produced in the tissues through reduction [19], may be responsible for their side effects and antileishmanial action.

Studies of the mechanism of cytotoxicity of the trivalent tartar emetic drug suggest that Sb(III) compromises thiol homeostasis through depletion of intracellular glutathione and inhibition of glutathione reductase [22]. Then, Sb(III) enhances oxidative stress and leads to apoptosis through increase of ROS [22, 62–64].

Further evidence that pentavalent antimonials exert toxicity through induction of oxidative stress was obtained by Kato et al. [65], who demonstrated the protective effect of the antioxidant ascorbic acid during antimonial chemotherapy in a murine model of visceral leishmaniasis.

30.4 Gold Complexes Against Leishmaniasis

Auranofin (Fig. 30.3) is a US Food and Drug Administration-approved drug used therapeutically for rheumatoid arthritis. It is the prototypical gold drug with remarkable broad-spectrum medicinal properties, inspiring the development of other Au(I) and Au(III) compounds [66]. More recently, the awarding of orphan-drug status to auranofin for the possible treatment of amebiasis (caused by *Entamoeba histolytica*, an intestinal protozoan parasite) has significant global health implications in developing countries [67]. Transcriptional profiling and thioredoxin reductase assays suggested that auranofin targets the *E. histolytica* thioredoxin reductase, preventing the reduction of thioredoxin and enhancing sensitivity of trophozoites to ROS-mediated killing. Auranofin also inhibits viral load in simian virus [68, 69]. In combination

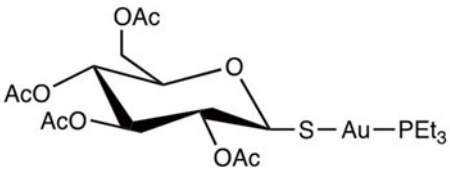

Fig. 30.3 Structure of Auranofin

with auranofin, administration of buthionine sulfoximine, an inhibitor of glutathione synthesis, under a highly intensified antiretroviral treatment was followed, after therapy suspension, by a significant decrease of viral RNA and DNA in peripheral blood as compared to pre-therapy levels.

Auranofin has also shown antiparasitic (malaria, leishmaniasis) activity, very likely arising from inhibition of parasitic enzymes involved in the control of the redox metabolism [11, 66]. The validation of TR as a key enzyme of *Leishmania infantum* polyamine-dependent redox metabolism and a target for antileishmanial drugs suggested that thiophilic agents besides Sb(III) could be effective enzyme inhibitors and potential antileishmanial agents. The X-ray crystal structure of the auranofin–trypanothione reductase–NADPH complex resolved at 3.5 Å resolution showed gold bound to the two active site cysteine residues of TR [70]. The thiosugar moiety of auranofin is located at the trypanothione binding site, suggesting that auranofin may inhibit TR through a dual mechanism. Enzymatic assays revealed that auranofin causes a pronounced enzyme inhibition and the drug kills the promastigote stage of *L. infantum* at micromolar concentrations.

An integrated in vitro and in vivo screening platform incorporating multiple leishmania life cycles and species probed a focused library of pharmaceutically active compounds for identification and prioritization of *bona fide* cytotoxic chemotypes toward leishmania parasites. Auranofin was confirmed as a potent cytotoxic antileishmanial agent and inducer of apoptotic-like death in promastigotes. Significantly, the antileishmanial activity of auranofin transferred to cell-based amastigote assays as well as in vivo murine models [71].

Auranofin may represent a prototype drug that can be used to identify signaling pathways within the parasite and host cell critical for parasite growth and survival. Indeed, a structurally diverse group of Au(I)/Au(III) compounds behave as highly effective inhibitors of *Leishmania infantum* TR, some being even more effective than antimonials [72]. Simultaneous consideration of TR inhibition and antiproliferative potency has identified appropriate candidates for further evaluation. The current results suggest a foundation for potential exploitation of gold-based complexes as chemical tools or the basis of therapeutics for leishmaniasis.

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