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EXPERT REVIEW

Mitochondria and Trypanosomatids: Targets and Drugs

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ABSTRACT The family *Trypanosomatidae*, flagellated parasitic protozoa, is responsible for important infectious diseases in humans: sleeping sickness, Chagas diseases and leishmaniasis. Currently, development of effective vaccines against these parasites remains an unrealized goal, and clinical management is based on chemotherapeutics. Cost, toxicity and resistance problems of conventional drugs result in an urgent need to identify and develop new therapeutic alternatives. The sound understanding of parasites, biology is key for identifying novel lead structures and new drug targets. This article reviews current knowledge about mitochondrial drug targets and existing drugs against *Trypanosoma* and *Leishmania*. In the past, several targets in trypanosomatid mitochondria (electron transport chain, kDNA and topoisomerases, tRNA import and fatty acid synthesis) have been identified. It has been suggested that inhibition of certain targets is involved in triggering apoptosis by impairment of mitochondrial membrane potential and/or production of reactive oxygen species. The inhibitory mechanism of approved drugs, such as pentamidine, nifurtimox, artemisinin and atovaquone, is described in parallel with others products from preclinical studies. In spite of the large amount of genetic information, the analysis of the phenotype of the trypanosomatid mitochondrion in different life stages will remain a useful tool to design new active compounds with selective toxicity against these parasites.

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ABBREVIATIONS

DIM	3,3′diindolylmethane
ETC	electron transport chain
FAS	fatty acid synthesis
FRD	fumarate reductase
gRNAs	short transcripts RNA
HAT	Human African Trypanosomiasis
kDNA	kinetoplastid DNA
MMP	mitochondrial membrane potential
NADH	nicotinamide adenine dinucleotide
NTDs	neglected tropical diseases
PCD	programmed cell death
RIC	rNA import complex
ROS	reactive oxygen species
TAO	*Trypanosoma* alternative oxidase
tRNA	transcription DNA

INTRODUCTION

The family Trypanosomatidae consists of a large group of flagellated parasitic protozoa causing infections in humans and animals. The most important infectious diseases in the man are caused by *Trypanosoma* species (sleeping sickness and Chagas diseases) and *Leishmania* (leishmaniasis). Most affected by these parasitic illnesses are the low-income populations of developing countries in tropical and subtropical areas of the world. Due to the fact that these diseases are less often treated and only limited research funding is spend, these have been considered as neglected tropical diseases (NTDs) (1).

Despite years of effort, the development of an effective vaccine against these protozoal parasites remains an

unrealized goal. Clinical management of diseases caused by trypanosomatids are still based on chemotherapeutics. However, conventional drugs are far from satisfying the current demands of endemic populations due to their cost, toxicity and resistance problems, resulting in an urgent need to identify and develop new therapeutic alternatives. The identification of novel agents requires a sound understanding of the parasites biology at both cellular and biochemical level. The genomic and proteomic advances together with bioinformatic tools generated in some details new information of general biological interest. The protozoa are also of interest from the cell biology point of view since they possess special cytoplasmic structures and organelles. Studies revealed that unique metabolic pathways exist in these organelles thereby opening new possibilities for the identification of new drug targets and new drugs (2).

One of the most fascinating organelles of vital importance to survival of protozoal cells are mitochondria, which include several drug targets. The past decade have given new impetus to mitochondrial research due to the diversity of findings on their structure and function in various cell types including protozoal and malignant mammalian cells (3,4). Experimental studies about the mechanism of action of the major antiprotozoal drugs suggest that currently protozoal mitochondria can be considered as one of the most fascinating targets inside protozoal organisms. Several scientific reviews in this field have contributed to a better understanding of mitochondrial function (5,6) and drug inhibitory action (7–9). *Trypanosoma* and *Leishmania* parasites are the main subject of this article. These protozoa exhibit a wide variation in the development of the mitochondrial organelle, which will be described according to the available information about trypanosomatid mitochondrial targets and related drugs.

TRYPANOSOMATID MITOCHONDRIA

General Characteristics of Trypanosomatid Mitochondria

The special characteristics of these pathogenic protozoa is that they contain typical mitochondria as a single organelle in comparison with mammalian cells possessing a hundreds to thousands mitochondria. Therefore, the proper function of the single mitochondrion in protozoal parasites is very vital compared with cells from mammals with numerous mitochondria because the presence of multiple mitochondria ensures compensation for functionally impaired ones. However, for organisms with a single mitochondrion no such choice exists and survival depends on proper function of this single organelle (10).

Ultrastructure

The ultrastructure of mitochondria in trypanosomatids is usually peculiar in comparison to that in multicellular organism, with respect to density of the matrix as well as number and shape of the cristae. Depending on environmental and nutritional resources available, the mitochondrion can fill up to 12% of the cell volume. The fine structure of mitochondria may vary depending on the genus and species of parasites, but generally the mitochondrion is distributed in branches under the subpelicular microtubes. In addition, the higher dilated regions of the mitochondrion contain the kinetoplastid DNA (kDNA), which is the most unusual structure in the organelle (6,11).

Kinetoplastid DNA

A small but essential part of proteins are encoded and produced in the mitochondria, which represents generally specific components of the respiratory chain and the mitochondrial translation machinery (12). The mitochondrial genomic information is contained in kDNA, which represents about 30% of the total cellular DNA. Their morphologic structure appears as a disc-like shape in the matrix and consists of small circular duplex molecules of a uniform size that are roughly 0.45 μm long, corresponding to 1440 base pairs and 0.94×10^6 Da of molecular weight (5,13). The kDNA is composed of two classes of circular molecules of different sizes, the maxi- and minicircles. Approximately, 50 copies of maxicircle DNA with a size between 20–40 kb depending on the species are found; while minicircles are present in 5,000 and 10,000 copies per organelle with a size smaller than 2.5 kb in most trypanosomatid species (14).

Functional Proteins

The proteins of mitochondria come from two sources. In most cases more than 95% are encoded in the nucleus, synthesized in the cytosol and post-translationally imported into mitochondria. The number of mitochondrially encoded proteins is small (about 18) and the expression of these proteins requires processes (mitochondrial translation), which are either unique for trypanosomes or show significant differences to other organisms. The maxicircles that establish 10% of the mass of the network are structurally and functionally analogous to the mitochondrial DNA of other organism. Studies revealed that these maxicircles encode 13 proteins of known identity: cytochrome *b*, subunits I-III of cytochrome oxidase, subunit 6 of the adenosine triphosphate synthase, six units of the reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase, a ribosomal protein and five open reading frames of unknown function.

The other 90% of the mass correspond to minicircles, which are heterogeneous in sequence and encode the majority of short transcripts called gRNAs (5).

Mitochondrial Activities

The life cycle of *Trypanosoma* and *Leishmania* are highly complex since the parasites exist in different morphological states. *Trypanosoma* species pass through three different forms: epimastigote (insect vector), trypomastigote (infective form) and amastigote (intracellular form); while *Leishmania* present two different stages: promastigote (insect vector) and amastigote (intracellular form). At every stage the parasites are metabolically well adapted to the respective compartment of their specific host. This specific adaptation is also reflected by the differential sensitivities of parasites to conventional drugs in their different life stages. The cell biological and biochemical processes in each state clearly indicate the presence of distinguishable stage-specific metabolic steps (15–17).

Trypanosomatidae species have mitochondria with common characteristics and active respiration is required for survival. Nevertheless, the mitochondrial activities in the certain life cycle stages can be very different. An example are bloodstream *T. brucei* cells that possess mitochondria, which cannot perform oxidative phosphorylation (5). In spite of the fact that the respiratory chain of bloodstream forms of *Trypanosoma* is not functional, drugs with mitochondrial targets, such as pentamidine, killed also the bloodstream form of *T. brucei* at higher concentrations (18).

In the case of *Leishmania* parasites, the basic studies (19) and inhibitor evaluations (10,20) have been performed with the promastigote form. Recently, Chakraborty and collaborators suggested that amastigote mitochondria from *Leishmania* are less dependent on respiratory energy (21). This might be the reason for the survival of amastigote cells within phagolysosomes where apparent hypoxic conditions persist. The authors suggest that amastigotes were devoid of complex IV activity and failed to consume oxygen (21). Experimental data have been presented that the amastigote failed to retain the activities of complex I and II, and as consequence to display oxidative phosphorylation (21). Some authors showed that an increased mitochondrial activity may play a crucial role in the survival of amastigotes inside host cells (22,23). Nevertheless, more complete studies are required to better understand the processes in mitochondria of *Leishmania* parasites.

Perspectives

In the last years, the increase of basic knowledge about mitochondria in trypanosomatids drew increased interest on this organelle as fascinating drug target. However, also many new interesting questions arose from basic research on mitochondrial structure and function in trypanosomatids, such as consequences of stage-specific activity, the differences with respect to mammalian mitochondria, as well as the unique mitochondrial features found in these protozoa. The intensive study of basic functions and effects of inhibitors in the different *Trypanosoma* and *Leishmania* species will be useful to understand the mechanism of antitrypanosomal drugs, the specific-sensitivity of species and can contribute to the design of new and potent therapeutic compounds.

Currently, studies of trypanosomatid mitochondria are scarce compared with other protozoa, such as Apicomplexa (*Plasmodium* and *Toxoplasma*). However, the knowledge so far obtained has demonstrated that the features of trypanosomatid mitochondria make them a promising target for the development of new drugs against *Leishmania* and *Trypanosoma* parasites.

MITOCHONDRIAL TARGETS

The structure and function of mitochondria in trypanosomatids has peculiarities compared with mammalian mitochondria. Below, the prominent functional differences of trypanosomatid mitochondria as drug targets are described.

Electron Transport Chain

In general, the electron transport chain (ETC) in mammalian mitochondria is composed by four integral electron transfer complexes in the inner mitochondrial membrane: Complex I (NADH:dehydrogenase ubiquinone, E.C. 1.6.5.3), Complex II (succinate: ubiquinone oxidoreductase, E.C. 1.3.5.1), Complex III or cytochrome bc_1 (ubiquinol: cytochrome c^{3+} oxidoreductase, E.C. 1.10.2.2) and Complex IV (cytochrome c^{2+} oxidase, E.C. 1.9.3.1), with ubiquinone (Coenzyme Q) and cytochrome c functioning as mobile electron carriers between the complexes (7). The proton gradient produced by electron transport drives the F_1/F_0 ATPase (Complex V) in a coupled process, which is termed oxidative phosphorylation (24).

In protozoa, the ETC has particularities that make it a promising target. One of them is an alternative complex I NADH:quinone oxidoreductase that is rotenone-insensitive (19,25). Studies demonstrated that an enzyme corresponding to the mammalian rotenone-sensitive complex I may be absent or not very active. The characteristic rotenone-insensitive NADH:quinone oxidoreductase has been observed in *Trypanosoma* and *Leishmania* and has no counterpart in humans (19,25). In *P. falciparum* parasites the genome sequence revealed a single subunit NADH dehydrogenase, which is incapable of proton pumping, serving mainly to

regenerate NAD^+ and reduce ubiquinone. Since parasites possess only one NADH dehydrogenase gene in the total DNA, it may be essential for the survival and thus could be an interesting drug target (26).

Basic biochemical studies suggested that in protozoa a classical complex II is present. Since the parasite has a limited electron transport between complexes I-III, succinate might be a primary electron donor for energy production. However, in parasites the succinate can be recycled from fumarate by fumarate reductase (FRD). This enzyme has been found in *Leishmania* (27) and *Trypanosoma* (28). Since this enzyme is absent from mammalian mitochondria, it could potentially be an important target for drugs against these parasites.

First efforts to target the ETC of protozoal parasites were directed to complex III or cyt bc_1 complex. Analysis of the amino acid sequence of cytochrome *b* revealed differences in putative ubiquinone interaction sites with respect to the mammalian protein, which are an attractive target for antiprotozoal drugs (29).

Complex IV is composed by more than 14 subunits, which has three mitochondrially encoded subunits and all the others are nuclear encoded subunits (30,31). One of the nuclear encoded subunit of this cytochrome c oxidase in *Leishmania* and *Trypanosoma*, plays a relevant role in mitochondrial function and has been correlated with infectious stages of trypanosomatids. These protein were reported to be present in *Leishmania donovani* and *Trypanosoma brucei* and were named Ldp27 (24) and Tb11.0400 (32), respectively.

Alternative Oxidase

One interesting peculiarity in the ETC is the presence of an alternative oxidase, which can be found in *Trypanosoma brucei* bloodstream form and a reduced level in the procyclic form. It appears that the corresponding enzyme in mammals has been lost during evolution (33,34). This type of *Trypanosoma* alternative oxidase (TAO) acts as a terminal electron acceptor in the mitochondrial ETC, which have been considered to be a non-proton pumping oxidase which is unlikely to participate in generating a proton motive force across the membrane of *Trypanosoma* mitochondria (7).

kDNA and Topoisomerases

Among important functions of mitochondria from trypanosomatids the mitochondrial replication, as well as other involved factors in this process have been described. During kDNA replication the DNA content of the network doubles and the progeny network partitions into two daughter cells. This occurs approximately synchronously with nuclear DNA synthesis. DNA topoisomerase II from kinetoplastid parasites has been implicated in the process, where it decatenates the kDNA network prior to replication of minicircles (14). In recent years, the mitochondrial DNA topoisomerases from parasites have been the focus of several studies since they may provide a target for new antiparasitic chemotherapy.

Some distinguishing features that differentiate the parasitic enzyme from its prokaryotic and eukaryotic counterparts have been identified (35). In the study of the structural determinants the recent detection of substantial differences between *Trypanosoma* and *Leishmania* mitochondrial DNA topoisomerases with respect to their homologues in mammals has provided a new effective target (36). Two classes of drugs that interfere with topoisomerase activity have been described. The topoisomerase inhibitors compete with ATP for binding to the catalytic domain of the topoisomerase and thus interfere with its function. In contrast, topoisomerase poisons stabilize the topoisomerase-DNA complex and result in DNA breakage (37). The second type of topoisomerase inhibitors is most frequently described in literature.

Recently, it has been reported that *Leishmania* topoisomerase I enzyme, that normally is found in the nucleus, is also located in mitochondria and might play an important role in kDNA metabolism (35). This conclusion has been deduced from camptothecin, a potent antitumor agent, which can trap topoisomerase I-mediated cleavable complexes in mitochondria of *Trypanosoma* and *Leishmania* parasites (38).

tRNA Import

Mitochondrial tRNA import occurs in many protozoa, in plants, some fungi and in a few invertebrates (39). In most organisms, which import tRNA, not all mitochondrial tRNA genes were lost. The situation in trypanosomatids is unusual since they have lost the entire set of their mitochondrial tRNA genes. It has been shown that in these organisms, tRNAs are imported from cytosol (39,40). Kinetoplastid protozoa have developed specialized systems for importing nucleus-encoded tRNA into mitochondria (41). The imported cytosolic tRNA was functional in mitochondrial protein synthesis and supported the repair of translational defects caused by a pathogenic point mutation in the mitochondrial genome. A large multiprotein aggregate (RNA import complex, RIC) from mitochondria of *Leishmania* was demonstrated to import tRNAs into phospholipid vesicles in an ATP-dependent manner, and is currently the only mitochondrial import complex described in a protozoal system (42).

The tRNA import has not been detected in any vertebrate species to date and, therefore, offers a novel potential target for a chemotherapeutic attack in trypanosomatids.

Fatty Acid and Sterol Synthesis

The fatty acid synthesis (FAS) in trypanosomatid parasites is essential for their survival and is different from that observed in higher eukaryotes (43,44). The recently completed genome sequence analysis of trypanosomatids indicated that these organisms are able to make fatty acids and it was shown that there is a type II FAS pathway, which takes place in the mitochondrion (44,45). Evidence from other organisms suggests that mitochondrially FAS is required for efficient respiration, but the exact relationship remains unclear (46).

Leishmania and *Trypanosoma* parasites produce ergosterol-related sterols by a biosynthetic pathway similar to that operating in pathogenic fungi and their growth is susceptible to sterol biosynthesis inhibitors. Thus, inhibition of squalene 2,3-epoxidase, 14-alpha-methylsterol 14-demethylase and delta(24)-sterol methyl transferase cause a depletion of normal sterols and an accumulation of abnormal amounts of sterol precursors with cytostatic or cytotoxic properties (47).

Mitochondrial Membrane Potential

Although the mitochondrial membrane potential (MMP) itself is not a single protein target but rather mitochondrial function, maintenance of proper MMP is essential for the survival of cells (48). Variations of the MMP can be a consequence of diverse events, such as the inhibition of ETC (decrease), block of ATP synthase (increase), stimulation of uncoupling proteins (decrease) or permeabilization of the inner membrane (decrease). As mentioned previously, a primary component supporting mitochondrial function is the successful operation of the ETC. Interestingly, in *Leishmania* inhibitors of complex II and III cause a dissipation of MMP as expected from mammalian mitochondria (10). Intriguing is the finding that the mammalian complex I inhibitor rotenone caused hyperpolarization in *Leishmania* mitochondria although these protozoa possess a rotenone-insensitive NADH: quinone oxidoreductase (not involved in proton translocation for MMP buildup) and in mammalian mitochondria rotenone causes a decrease of MMP (10). This suggests alternative activities for rotenone in *Leishmania* mitochondria.

Reactive Oxygen Species

In addition, dysfunction of ETC can result in excessive release of reactive oxygen species (ROS) from mitochondria. The deviation of electrons from the mitochondrial complexes is the primary source of endogenous ROS (49). Care must be taken for the interpretation of most published cellular ROS data obtained by the use of fluorescence dyes. A typical dye dichlorodihydrofluoresceine ($DCFH_2$), which neither directly reacts with $O_2^{\bullet -}$ and H_2O (most important ROS) but strongly with redox active iron, is often used (50,51). Taken into account this methodical limitation, in trypanosomatids complex II inhibition appears to be the prime area of $DCFH_2$ oxidation, which possibly is related to ROS production. In higher eukaryotes the major mitochondrial sites for ROS production are complex I and III (52), which mark another difference between trypanosomatids and mammals.

Apoptotic Death

Besides the bioenergetic, biosynthetic and metabolic roles of mitochondria inside cells their involvement in apoptosis regulation has become a major research focus (48). The molecular mechanisms associated with programmed cellular death (PCD) or apoptosis have been widely explored in mammalian cells. Currently, PCD mechanisms are known to be operative in kinetoplastid parasites of the genus *Trypanosoma* and *Leishmania*, but not yet precisely understood (20,53). Basic steps how protozoal mitochondria are involved in the apoptotic pathway have been demonstrated: (a) loss of membrane potential and ATP levels, (b) increase of H_2O_2 and superoxide radical generation, (c) increase of intracellular Ca^{2+} levels, and (d) initiation of apoptotic changes.

Perspectives

As shown, different possible targets can be identified in trypanosomatid mitochondria, which are related to their unconventional morphology, composition and functionality in comparison with mammalian mitochondria. Although the bioenergetic function of the organelle in trypanosomatids is limited, it might be essential for parasite survival. In addition, the application of multi-targeted drugs in this organelle represents an innovative approach to addressing chemotherapy-induced drug resistance. In this approach a single compound endowed with a multifunctional profile is able to simultaneously inhibit the activity of two or more mitochondrial targets. Per example, phenyl-phenalenone inhibited succinate- and NADH-cytochrome *c* reductase, as well as the purified FRD (54) and 3,3′diindolylmethane (DIM) stabilizes the formation of topoisomerase I-DNA cleavable complex, inhibits the complex V activity and increased the ROS generation (55).

Following the same rational approaches to design new drugs, the similarities between *Trypanosoma* and *Leishmania* parasites should permit that one drug can act on both parasites. The application of a multifunctional drug against different protozoal diseases would be useful in endemic areas with both infections and where differential diagnostics is not available to all infected patients.

CHAGAS DISEASES

General Characteristics

Trypanosoma cruzi is the pathogen that causes Chagas disease, which is endemic in American countries. The parasite attacks people living in remote rural areas that lack diagnostic facilities and good health records or statistics. Therefore, sufficient epidemiological information about its prevalence has not always been accessible. So far available epidemiological data show that the diseases represent a major public health problem in South America, affecting at least 8–9 million of people with more than 25 million at risk of infection (56). The chronic phase typically occurs 10 to 20 years after infection by the parasite and affects 10 to 30% of those infected. Cardiac and gastrointestinal pathologies are the most common manifestations of the chronic disease (17).

Currently Used Drugs

The available therapy to Chagas diseases is based on two agents introduced in the market in the 1970s: benznidazole (Figure 1, Compound 1) and nifurtimox (Figure 1, Compound 2), belonging to the class of nitroaromatic compounds. Their efficacy is limited to the disease's acute phase and only to some pathogen strains. Moreover, side effects, such as anorexia, vomiting and diarrhea impose limitations for their use in chronic patients (57,58).

Drugs Targeted to Mitochondria

Relevance of mitochondria in *T. cruzi* parasites as target has been demonstrated by the activity of different inhibitors, which caused (a) functional and structure alterations of mitochondria, (b) loss of ATP production by oxidative phosphorylation and (c) onset of apoptosis in parasites.

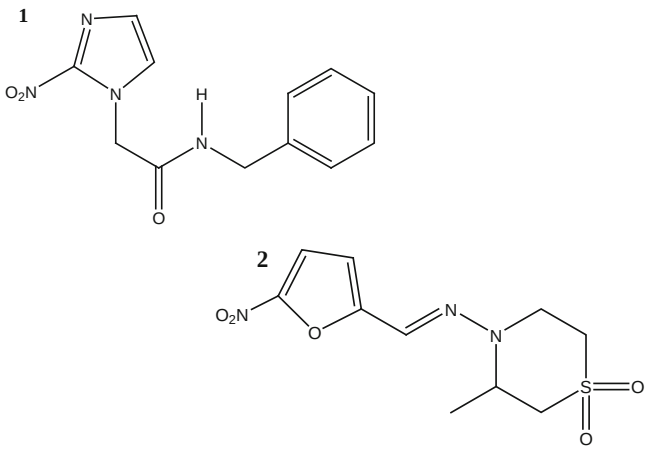

Fig. 1 Chemical structures of current drugs against Chagas disease. 1: Benznidazole; 2: Nifurtimox.

Nifurtimox, a nitrofurane derivative used in the clinical treatment of Chagas diseases, is believed to exert its activity against *T. cruzi* through the bioreduction of the nitro-group to a nitro-anion radical, which undergoes redox-cycling with molecular oxygen. The ability of nifurtimox and derivatives to induce oxidative stress due to inhibition by NAD(P)H-dependent dehydrogenases with subsequent impairment of MMP was reported, although the mode of action of nifurtimox remains an open subject (59).

Different mitochondrial targets in *T. cruzi* have been identified by the inhibitory action of compounds. Thiolactomycin and a number of its analogues showed inhibition against *T. cruzi* and *T. brucei*. This compound is an inhibitor of type II-ketoacyl-acyl-carrier-protein synthase which is found in plants and most prokaryotes, but not an inhibitor of type I fatty acid synthase in mammals (60). Etoposide was shown to be a potent inhibitor of topoisomerase II, which promotes the cleavage of minicircle DNA in trypanosomatids. After etoposide treatment, the residual minicircle catenanes have a sedimentation coefficient, which is only 70% that of controls and by electron microscopy the networks appear less compact. Double-size networks are characteristic dumbbell-shape forms which usually arise in final stages of network replication. After etoposide treatment these are replaced by aberrant unit-size forms (14,61).

Morphological Studies of Mitochondrial Dysfunction

For most of compounds assayed against *T. cruzi*, only ultrastructural studies on mitochondria have been described. The synergic anti-proliferative effect of lysophospholipid analogues (edelfosine, ilmofosine and miltefosine) and ketoconazole against *T. cruzi* was reported. These studies describe effects against epimastigotes and intracellular amastigotes and suggested that this drug combination targets also mitochondria, possibly by interference with lipid metabolism (62). The arylimidamide, a compound known as DB766, exhibits strong activity and excellent selectivity for bloodstream trypomastigotes and intracellular amastigotes of *T. cruzi*. By fluorescent and transmission electron microscopy analyses, the authors found that DB766 localizes in the DNA-enriched compartments and induces considerable damage to mitochondria (63).

Experimental evidence by ultrastructural studies about mitochondrially targeted products have been obtained for 2,4-dichloro-6-phenylphenoxyethyl diethylamine hydrobromide (64) and naphthoimidazoles (65). Also a variety of natural products have been explored as therapeutic alternatives against *T. cruzi*. An usnic acid from *Cadonia substellata* (66), the venom from *Bothrrops jararaca* (67) and *Crotalus viridis*

viridis were effective against all developmental forms of *T. cruzi* (68). Ultrastructural analysis revealed swelling of mitochondria under these conditions, which was confirmed by other assays, including the staining with rhodamine 123 (68).

The activity of the sesquiterpene elatol from the Brazilian red seaweed *Laurencia dendroidea* has been studied. Elatol showed a dose-dependent effect against epimastigote, trypomastigote, and amastigote forms of *T. cruzi*. Transmission and scanning electron micrographs demonstrated aberrant-shaped cells and prominent swollen mitochondria (69).

SLEEPING SICKNESS

General Characteristics

The sleeping sickness or Human African Trypanosomiasis (HAT) is caused by two different species of trypanosomes: *Trypanosoma brucei gambiense* in West and central Africa, and *Trypanosome brucei rhodesiense* in East Africa. Today, 60 million of people are exposed to HAT and it is estimated that 300,000 people are currently infected. *T. b. gambiense* infections have a chronic and protracted course, whereas *T. b. rhodesiense* is acute and can cause death in a matter of weeks or months. Both parasitic diseases are characterized by the presence of trypanosomes in central nervous system and the cerebrospinal fluid, which can cause fatalities if left untreated (16,56).

Currently Used Drugs

The treatment of first phase of sleeping sickness relies on suramin (Figure 2, Compound 1) and pentamidine (Figure 2, Compound 2). In the neurological phase melarsoprol (Figure 2, Compound 3) is active against *T. b. gambiense* and *T. b. rhodesiense;* whereas eflornithine (Figure 2, Compound 4) only can useful against *T. b. gambiense*. These drugs are the mostly antiquated, scarce, highly toxic and subject of parasite resistance (70,71).

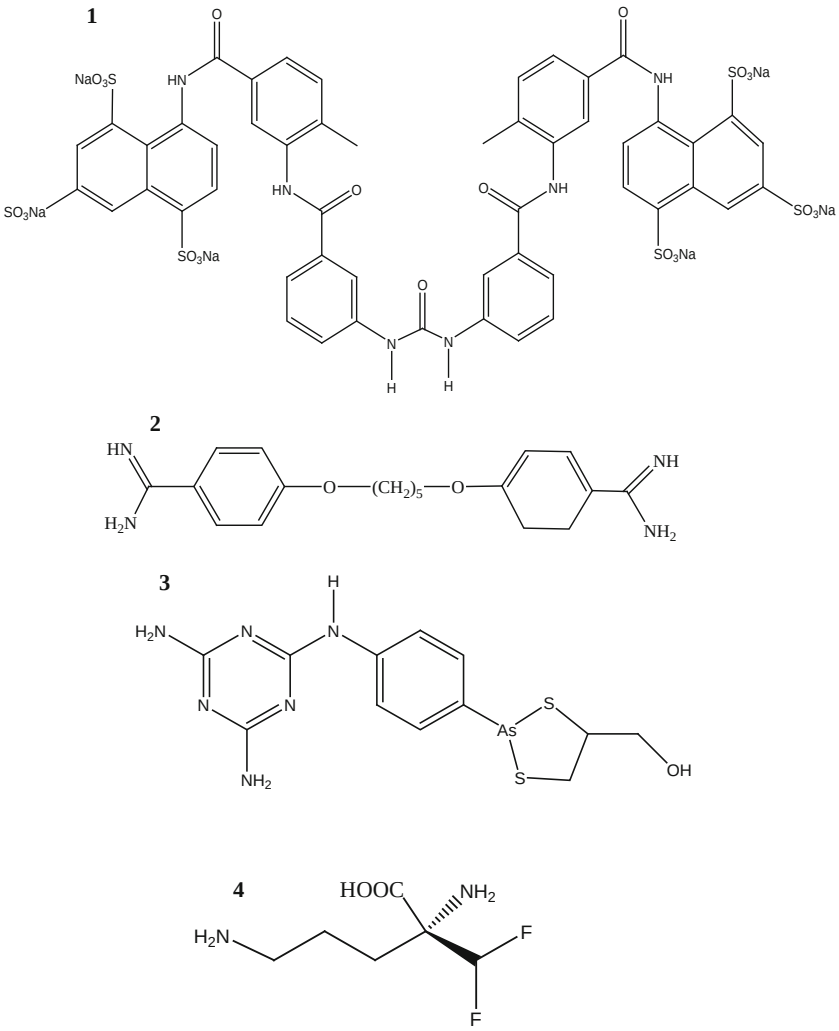

Fig. 2 Chemical structures of current drugs against sleeping sickness. 1: Suramin; 2: Pentamidine; 3: Melarsoprol; 4: Eflornithine.

Drugs Targeted to Mitochondria

A substantial number of anti-trypanosomatid drugs, including compounds clinically used and others under investigation, have the mitochondrion as at least one of their targets. Pentamidine is a dicationic drug and has been used for the last 50 years to treat African trypanosomiasis as well as cases of antimonial-resistant leishmaniasis (72). Several targets have been described for pentamidine action, but one of the most explored changes is the imbalance in the intracellular Ca^{2+} content, which collapses the MMP. In addition, pentamidine were found to promote linearization of *T. equiperdum* minicircles from the kDNA networks (61). Resistance to pentamidine has been described for *Trypanosoma* (73) and *Leishmania*. Biochemical studies revealed that resistant clones showed a decrease in the MMP. The basis of this coordinated downregulation of the expression of several enzymes causing resistance and survival of parasites is unknown. This regulation could be correlated with the decreased activity of numerous mitochondrial dehydrogenases. The decreased in MMP in resistant strains are also correlated with a decreased drug accumulation in the mitochondrial compartment, as well as changes in their volume (74,75). Pentamidine resistant parasites showed a cross-resistance to other toxic diamine derivatives (75). DB75, a structural analogue of the aromatic diamine drug pentamidine, has shown an activity against *T. brucei*, which is also accompanied by a collapse of the MMP via inhibition of the mitochondrial F_1/F_0-ATPase (76).

Notably, two recent reports on the TAO inhibitor ascofuranone (77,78) have shown that this compound has therapeutic properties in mice infected with either *T. brucei* or *T. vivax*. Cordycepin and quercetin, drugs under preclinical studies, showed strong effects against *T. b. gambiense*. Experiments demonstrated that the treatment with these compounds promoting topoisomerase II kDNA cleavage resulted in programmed cell death (79–81).

Morphological Studies of Mitochondrial Dysfunction

To our knowledge, no relevant studies exist.

LEISHMANIASIS

General Characteristics

The spectrum of diseases known as leishmaniasis is caused by various species of *Leishmania*. About 12 million cases of leishmaniasis exist worldwide and 350 million people live at risk of infection in 88 tropical and subtropical countries. The clinical manifestations include three major groups of disorders: cutaneous leishmaniasis, mucocutaneous leishmaniasis and visceral leishmaniasis (kala-azar), which range from potentially disfiguring cutaneous infection to often fatal visceral diseases (15).

Currently Used Drugs

Treatment of leishmaniasis is still complicated since different causative *Leishmania* species and various clinical manifestations exist. Nowadays, there are about 25 compounds and formulation available that show antileishmanial effects, but only a few are classified as antileishmanial drugs for humans. First-line treatment is based on drugs containing pentavalent antimony: meglumine antimonate (Glucantime) (Figure 3, Compound 1) and sodium stibogluconate (Pentostam) (Figure 3, Compound 2); while as second-line drugs amphotericin B (Figure 3, Compound 3), pentamidine (Figure 2, Compound 2) and paromomycin (Figure 3, Compound 4) can be used. Recently, miltefosina (Figure 3, Compound 5), the first oral dug to visceral leishmaniasis has been approved (15).

Drugs Targeted to Mitochondria

In *Leishmania*, mitochondria as target have been more extensively explored, and the results demonstrated the importance of this organelle to survival of parasites. Experimental evidences showed that some of second-line drugs target mitochondria. For example, amphotericin B causes a permeability of membranes with a rapid decrease of MMP followed by a simultaneous induction of plasma membrane permeability (82) and also pentamidine collapses the MMP, as explained previously.

Benzophenone-derived bisphosphonium salts were synthesized and assayed for lethal activity on *Leishmania*. The results suggest that the compound targets complex II of the parasital respiratory chain based on findings of: (a) a dramatically swollen mitochondrion in treated parasites, (b) fast decrease of cytoplasmic ATP, (c) a decrease of the MMP, and (d) inhibition of the oxygen consumption rate using succinate as substrate (83).

Artemisinin, a sesquiterpene lactone isolated from *Artemisia annua*, is an established anti-malarial compound that showed anti-leishmanial activity in both promastigote and amastigote. The anti-leishmanial action was mediated by apoptosis and loss of MMP (84). Activity of artemisinin against *T. cruzi* and *T. brucei* was also observed which could result from mitochondrial damage as well (85).

The hydroxynaphthoquinone atovaquone showed activity against *L. infantum* with IC_{50} values of 15.2 μg/mL (86). Since 1940s, hydroxynaphthoquinones have been suspected to act as ubiquinone competitors, although the first approved drug and best known example is atovaquone. This derivative was initially developed as an antimalarial

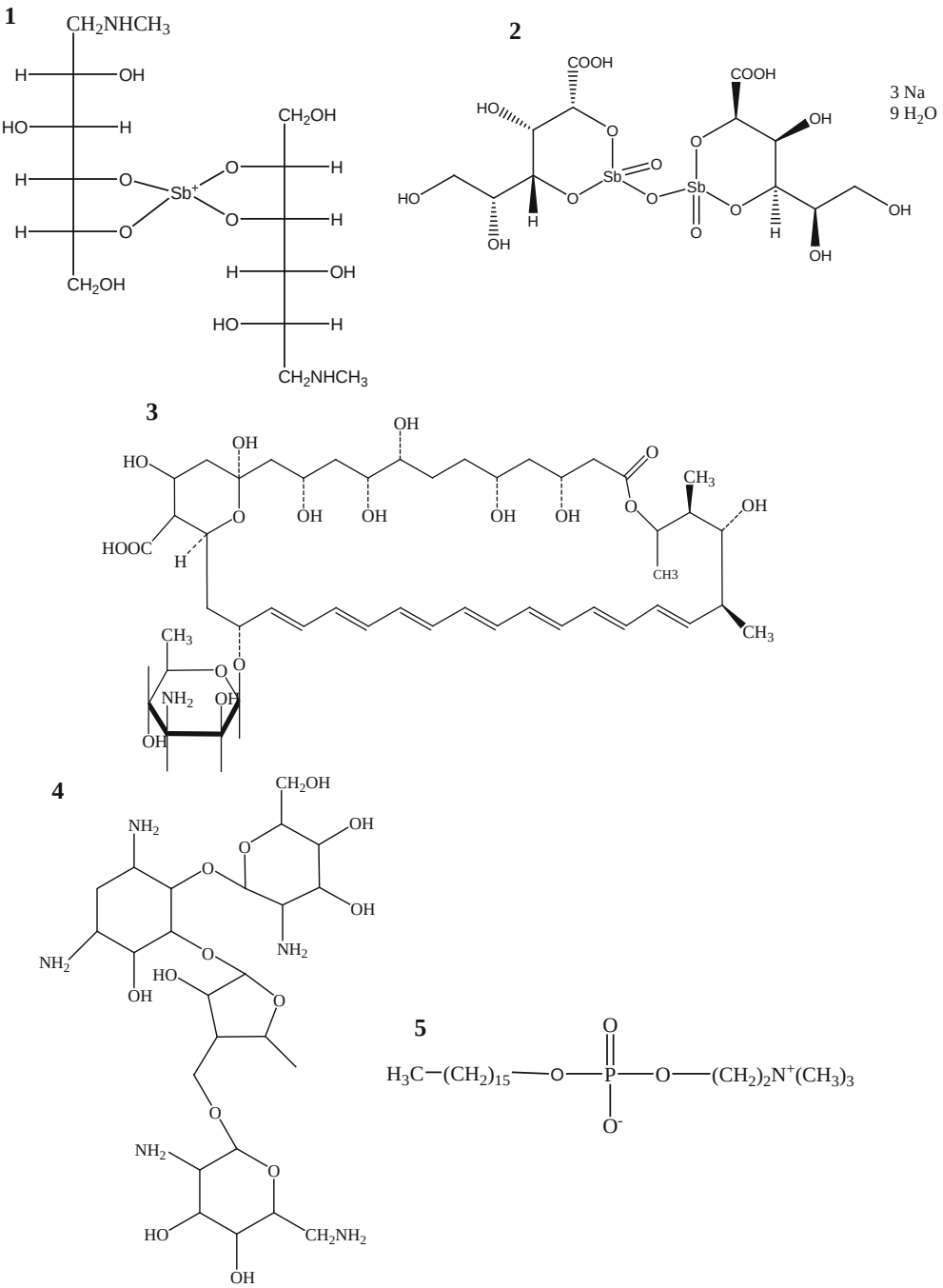

Fig. 3 Chemical structures of current drugs against leishmaniasis. 1: Meglumine antimoniate; 2: Sodium stibogluconate; 3: Amphotericin B; 4: Paromomycin; 5: Miltefosine.

compound and inhibited the parasite mitochondrial cytochrome bc_1 complex at 1000-fold lower concentration than the corresponding complex in mammalian mitochondria (87). Nevertheless, low sensitivity of *L. tarentolae* wild type against atovaquone and also *Leishmania* strains resistant to atovaquone have been observed (86). Several studies of the atovaquone-resistance mechanism were reported for the *Plasmodium* parasite, which revealed that the resistance is correlated with mutations of five amino acids from the cytochrome *b* subunit. Three of the involved residues (I258, Y268 and L271) are absolutely conserved in cytochrome *b* from mammals and protozoa; while the other involved residues (F267 and K272) were different between the parasite and vertebrate proteins (87). However, so far no direct evidence exists that in trypanosomatids atovaquone resistance is related to cytochrome *b* mutations. Recent advances have shown that a modification of side chain of atovaquone might overcome resistance in plasmodia (88).

The chalcones, including licochalcone A and others derivatives obtained synthetically, exhibit potent antileishmanial activity. Experimental studies showed that these compounds destroy the ultrastructure of mitochondria in *Leishmania* parasites and inhibited the FRD (89). The enzyme was also inhibited with aurones (90) and phenylphenalenone phytoalexins isolated from *Musa acuminate* (54).

Tafenoquine, an 8-aminoquinoline analogue of primaquine, which is currently under clinical trial (phase IIb/III) for the treatment and prevention of malaria, may represent an alternative treatment for leishmaniasis. Their mechanism of action against *Leishmania* parasites includes mitochondrial dysfunction through the inhibition of cytochrome c reductase (respiratory complex III) thereby decreasing the oxygen consumption rate and depolarizing the mitochondrial membrane potential. This was accompanied by increased ROS production, elevation of intracellular Ca^{2+} levels, concomitant nuclear DNA fragmentation and leads to an apoptosis-like death process (91).

In *L. donovani*, treatment with luteolin and quercetin, which are plant-derived flavonoids that occur abundantly in our daily diet, induce the loss of both maxicircles and minicircles and resulted in the formation of dyskinetoplastid cells. The loss of mitochondrial DNA causes alteration in mitochondrial structure and consequently the reduction in the activities of complexes from ETC and associated with a decrease in mitochondrial ATP production (81). In addition luteolin and quercetin inhibited the growth of *L. donovani* due to the promotion of topoisomerase II linearization (81). The camptothecin and DIM are compounds that directly stabilize the formation of topoisomerase I-DNA cleavable complex in *Leishmania* cells and increased the ROS generation. Interesting, DIM also severely inhibit the Complex V activity, which comprises of the F_0/F_1 synthase (55).

Among natural products that inhibit *Leishmania* parasites and cause depolarization in MMP, generate ROS in cells and cause PCD, the following compounds are worth mentioning: racemoside A, a purified water-soluble steroidal saponin from the fruits of *Asparagus racemosus* (92); withaferin A, a steroidal lactone isolated from leaves of *Withania somnifera* (93); the landrace Blanga Mahoba of *Piper betle* (94) and the berberine, a quarternary isoquinoline alkaloid (95).

Morphological Studies of Mitochondria Dysfunction

Furazolidone is a nitrofuran derivative that induces severe ultrastructural alterations to parasite mitochondria in *L. chagasi*, *L. braziliensis*, *L. major* and *L. amazonensis* (96). In *Leishmania* parasite 3-substituted quinolines (97), sterol methenyl transferase inhibitors (98) and triazoles-pyrimidine complexes (99) caused alterations in mitochondrial structure.

CONCLUSIONS

Today, the key role of mitochondria as drug target has been recognized due to extensive studies in mammalian and yeast mitochondrial systems. Recently, this organelle in protozoal parasites is gaining more and more attention in morphological and physiological research. Impressive progresses in the

Table I Comparison of Mitochondrials Target in *Trypanosoma* and *Leishmania* with Respect to Mammalian Mitochondria and Currently Reported Drugs

Mitochondrial target	Comparison between parasite and mammalian mitochondria			Inhibitors	Reference
	Trypanosoma	*Leishmania*	Mammalian		
Complex I	Rotenone-insensitive		Rotenone-sensitive	–	–
Fumarate reductase	Succinate is recycled from fumarate		Absent	Licochalcona A	(89)
Complex III	n/a	Differences in putative ubiquinone interaction sites		Atovaquone	(86,87)
				hydroxy-naphthoquinone	(88)
				Tafenoquine	(91)
Alternative Oxidase	Trypanosoma alternative oxidase	n/a	Absent	Ascofuranone	(77,78)
nucleic acid	kDNA		mtDNA	Luteolin Quercetin	(100)
Topoisomerase II	Substantial differences between trypanosomatid DNA topoisomerase respect to homologues in mammals			Pentamidine	(61)
				Etoposide	(14,61)
				Luteolin Quercetin	(81)
tRNA import	tRNAs are imported from cytosol		Absent	–	–
Fatty acid synthesis	Type II fatty acid synthesis pathway		Type I fatty acid synthesis pathway	Thiolactomycin	(60)

n/a: no scientific information available

understanding of physiological processes in mammalian mitochondria have been made. However, still less is known about mitochondrial functions in *Leishmania* and *Trypanosoma* parasites. The knowledge about mitochondria in trypanosomatids is still incomplete and we are just at the beginning to understand the role of mitochondria in these protozoal cells.

A post-genomic view of the mitochondrial features in trypanosomatids parasites shows that despite the drastic reduction in physiological processes (based proteins coded by DNA) carried out in the mitochondrion, the adaptation of mitochondria to the different life stages of trypanosomatids is not yet understood. In addition, other functions attributed to mitochondria (amino acid metabolism, sterol biosynthesis, calcium homeostasis and ubiquinone synthesis) that have been studied in others protozoal parasites deserve special attention in trypanosomatids.

During the last decade an explosive increase in publications about mitochondria as drug target was observed for the following reasons: (i) it was recognized that mitochondria as organelles are important for survival of parasites (ii), the availability of purified enzymes and fractions from these parasites enabled detailed measurement of some basic mitochondrial functions, including oxygen consumption, changes in MMP, and other activities, (iii) the mitochondrion as apoptotic trigger received much attention, (iv) the development of parasite resistance to some drugs that target mitochondria stimulated an increased interest of researchers to understand the peculiarities of trypanosomatid mitochondria. A comparison of mitochondrial targets in *Trypanosoma* and *Leishmania* compared with mammalian mitochondria and some inhibitors have been summarized in Table I.

In our perspective, the introduction of multitargeted drugs is one of the best strategies to improve the efficacy, decrease the toxicity and could be a way to counteract the rapid development of resistance. At the same extent the activity of drugs in multiple parasites is a major concern in NTDs drug discovery and development. So far only a few efforts have been devoted to this direction.

As presented in this review, several targets have been identified in trypanosomatid mitochondria in part by analysis of the anti-trypanosomatid activity of different drugs. In our opinion a better understanding of the mitochondrial function in trypanosomatids in different life stages will strongly support current and future exploration new agents against *Trypanosoma* and *Leishmania* protozoa parasites.

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