

Contributions of Ultrastructural Studies to the Cell Biology of Trypanosomatids: Targets for Anti-Parasitic Drugs

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Abstract: Protozoan parasites cause disease in humans worldwide, and many fall into the genera *Trypanosoma* and *Leishmania*; these parasites are responsible for African trypanosomiasis, Chagas disease and the different forms of Leishmaniasis. Strategies for the development of new drugs against these protozoans have been based on their cell biology and biochemistry complemented by the use of electron microscopy. *Trypanosoma* and *Leishmania* have special organelles that are involved in metabolic pathways, which are very distinct from those in mammalian cells; these organelles are potential drug targets. Scanning and transmission electron microscopy can identify not only the target organelles but also alterations to the cell surface and ultrastructural changes that characterize distinct forms of programmed cell death.

Keywords: *Trypanosoma cruzi*, *Leishmania*, African trypanosomes, Chemotherapy, Ultrastructure.

1. INTRODUCTION

Parasitic protozoans of the genera *Trypanosoma* and *Leishmania* infect millions of people around the world. The diseases (African sleeping sickness, Chagas disease and leishmaniasis) caused by these parasites are in the group of 13 neglected tropical diseases classified by the World Health Organization. All of them occur in areas of extreme poverty and are forgotten by the government, while chemotherapy in patients is not economically attractive to pharmaceutical companies. Other factors that make this situation even worse are the lack of effective vaccines, the use of old, toxic drugs with variable efficacy and parasite resistance, the parenteral administration and the length of treatment [1-3].

Despite the lack of interest of different governments in eradicating these diseases, the trypanosomatids (even those not pathogenic to mammals) are the primary focus of various research groups around the world as important models for studying certain aspects of basic eukaryotic cell biology. Ultrastructural, physiological, biochemical and molecular approaches have shown that trypanosomatids (like other protozoan parasites) present special organelles, different metabolic pathways and special classes of enzymes. These features can be targeted for the development of new specific chemotherapeutic agents, including those isolated from natural compounds that can minimize the toxic effects on the host [3, 4-7].

In this review we will discuss how electron microscopy can help to identify the cellular locations of molecular targets of several drugs in different developmental stages of *Trypanosoma brucei*, *T. cruzi* and *Leishmania* spp. We will

also describe in detail the effects of drugs on the ultrastructure of the parasites.

2. ULTRASTRUCTURAL ASPECTS OF DRUG-TREATED PARASITES

Scanning and transmission electron microscopy have been frequently used in the study of drug-treated parasites. With the scanning electron microscope (SEM), changes in cell shape (shrinking, swelling, twisting), loss of the flagellum and cell membrane rupture can be observed. With the transmission electron microscope (TEM), the fine structure of the cell membranes and the different organelles can be observed (Fig. 1). Changes in composition of the organelles can be analyzed by cytochemical techniques, X-ray microanalysis or electron spectroscopy with loss of energy [8]. A brief explanation of the normal morphology of the parasites and the mechanism of action of the different classes of drugs will be presented.

2.1. Mitochondria

Trypanosomatids have only one highly ramified mitochondrion distributed throughout the protozoan body as revealed by observations of thick serial sections and of whole cells with high-voltage electron microscopy [8]. The mitochondrion is present in all developmental forms of the different trypanosomatids, and significant changes in the morphology of the organelle were observed in African Trypanosomes and in *Herpetomonas samuelpeessoai* depending on the metabolic activity of the parasites. The morphological differences observed included changes in the relative volume of the mitochondrion in relation to the whole cell, the presence of cristae and the expression of mitochondrial enzymes. Although no differences in mitochondrial structure were observed among the developmental stages of *T. cruzi* and *Leishmania*, differences in molecular composition cannot be ruled out and could be important to define new molecular targets.

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The most frequent effect of different chemotherapeutic agents on trypanosomatid mitochondria is swelling of the organelle, the intensity of which varies according to the type and concentration of the drug and the time of incubation [9, 10].

Cationic drugs, such as crystal violet, which are used to prevent transfusion-associated Chagas disease and aromatic diamidines were the first drugs to be used in chemotherapy against pathogenic trypanosomatids [11-15]. The effect of crystal violet on *T. cruzi* is due to enhancement of production of reactive oxygen species (ROS) that inhibit mitochondrial respiration and cause its swelling, which is more intense in trypomastigotes [14].

Aromatic diamidines (such as pentamidine) have been used with success for more than 60 years in the therapy and prophylaxis of African trypanosomiasis and antimony-resistant visceral leishmaniasis [16, 17]. They have been described as DNA binders but also inhibit multiple classes of enzymes such as proteases, topoisomerases, polymerases, and others [15]. More recently, new diamidine-related analogues were developed that present improved anti-parasitic activity against *T. cruzi* and *Leishmania* [18, 19]. In *T. cruzi*, some of the new compounds (classified as reversed amidines) presented a high level of activity against amastigotes and trypomastigotes both *in vivo* and *in vitro* [18-26]. Ultrastructural analysis showed that the different aromatic diamidines displayed similar effects in *T. brucei*, *T. cruzi* and *Leishmania*, characterized by the presence of swollen mitochondria presenting low electron-density structures, fragmentation of the membrane and crista and mitochondrial disruption [12, 13, 18, 20, 23, 24, 27-32].

T. cruzi and *Leishmania* mitochondria are altered when the parasites are incubated in the presence of sterol biosynthesis inhibitors (SBIs). Sterols are constituents of cellular membranes that are essential for their normal structure and function. The success of SBIs in the treatment of Chagas disease and leishmaniasis is mainly due to a particular aspect of sterol biosynthesis in these parasites: unlike mammalian cells and similar to fungi, some members of the Trypanosomatidae family synthesize a special class of sterols (i.e., ergosterol and other 24-methyl sterols) instead of cholesterol. Ergosterol biosynthesis depends on the presence of a specific enzyme, $\Delta^{24(25)}$ sterol methyltransferase (SMT), which is not found in mammalian cells, and the use of its specific inhibitors has potential as an effective chemotherapy [9]. Several drugs that interfere with sterol biosynthesis were used in studies with *T. cruzi*, *T. brucei* and *Leishmania*. These include statins, which act on the mevalonate pathway by inhibiting HMG-CoA reductase [33]; biphosphonates, which interfere with the isoprenoid pathway in the step catalyzed by farnesyl diphosphate synthase (FDS) [34]; zaragozic acid and quinuclidines, which are inhibitors of squalene synthase (SQS) [35]; allylamides such as terbinafine, which are inhibitors of squalene epoxidase [33]; azoles such as ketoconazole, fluconazole, itraconazole, voriconazole and posaconazole, which inhibit C14 α -demethylase; and azasterols, which inhibit SMT (Figs. 2A, C, D, H).

Morphological changes observed in the mitochondria of trypanosomatids can be explained by the presence of ergosterol in their membranes [36]. Ketoconazole produces an

intense swelling of the mitochondrion, which may occupy up to 80% of the cell volume in *T. cruzi* with loss of inner membrane organization and matrix contents [37]. The combination of ketoconazole and terbinafine, in addition to inducing swelling, also caused the formation of paracrystalline arrays within the matrix of the *Leishmania* mitochondrion [37, 38]. SQS inhibitors produce mitochondrial swelling, separation of the outer and inner membranes and the presence of myelin-like figures and concentric membranes in the mitochondrial matrix [39-42] (Fig. 2D). The same effects were observed after treatment of *T. cruzi* and *Leishmania* with SMT inhibitors [43-46] (Fig. 2A). No alterations in mitochondrion structure have been described in *T. cruzi* after incubation in the presence of miconazole or econazole [47].

Lysophospholipid analogues (LPA) are a class of compounds that have been developed as anticancer agents. Their mechanism of action is based on their ability to insert into the membrane lipid bilayer and to change properties such as permeability, fluidity and signal transduction. LPA also interfere with the synthesis, breakdown and modification of membrane lipids [48]. More recently, these compounds were also tested against *Leishmania* and *T. cruzi* [49]. Miltefosine, edelfosine and ilmofosine caused swelling of *Leishmania* mitochondria and the presence of concentric membranes in the mitochondrial matrix [50]. No alterations were observed when *T. cruzi* was treated only with edelfosine; however, combined treatment with LPA analogues plus ketoconazole induced mitochondrial swelling [51, 52].

Lapachol and β -lapachone are naphthoquinones isolated from *Tabebuia* spp. trees that show activity against viruses, tumoral lineages and pathogenic microorganisms [53]. The mechanism of action of these compounds involves the generation of free radicals, which promote mitochondrial dysfunction. The effect of naphthoquinones and their naphthoimidazole derivatives on mitochondrion structure are drastic, with an intense swelling and the presence of intramitochondrial vesicles [54-58] (Figs. 2B, E).

The polyamine biosynthetic pathway has been shown to be an effective target for treatment against diseases caused by protozoan parasites. In trypanosomatids, the inhibition of polyamine synthesis impairs the synthesis of trypanotone, which is essential for protection of the parasite against reactive oxygen species produced by the host [59]. Interference in the synthesis of polyamines with eflornithine or DFMO (difluoromethylornithine), a selective irreversible inhibitor of ornithine decarboxylase (ODC), induces changes in the differentiation process in *T. b. gambiense*. However, it was not effective against *Leishmania* or *T. cruzi* due to differences in the gene and an inability to produce significant amounts of putrescine, respectively [60-63]. Use of the putrescine analogue 1,4-diamino-2-butanone (DAB) caused inhibition of the proliferation of *Leishmania* and *T. cruzi* and severe damage to the mitochondrion characterized by swelling, fenestration and the presence of few cristae [64-68].

Alternative treatments have been introduced into the chemotherapy of parasitic diseases, and many studies based on the use of synthetic compounds, plant extracts, natural products and venoms have been published. The biological activity of natural products is mainly associated with the presence of compounds such as flavonoids, aromatic acids,

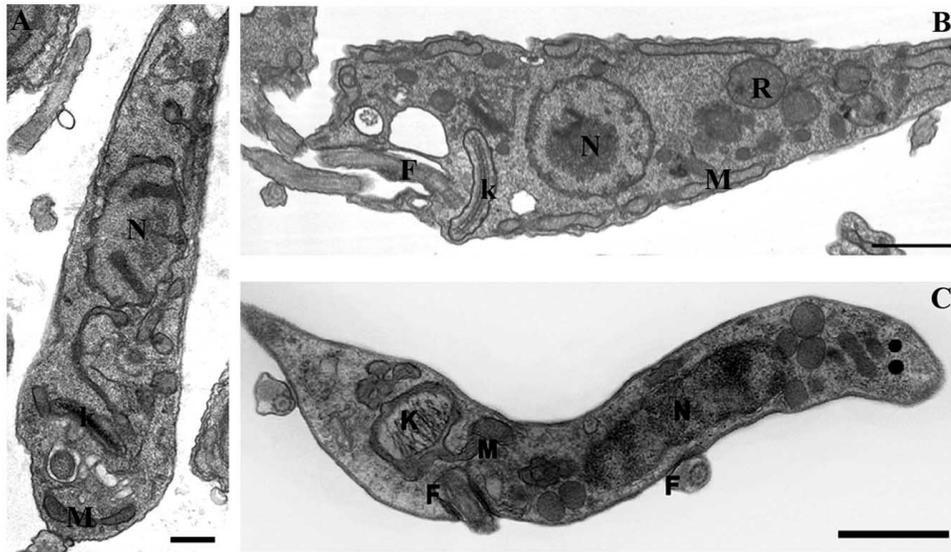


Fig. (1). Ultrathin sections showing untreated parasites presenting typical morphologies. (A) An *L. amazonensis* promastigote. (B-C) A *T. cruzi* epimastigote and a trypomastigote, respectively. N, nucleus; K, kinetoplast; M, mitochondrion; F, flagellum; R, reservosome. Bars: 1 μ m. (A) Courtesy of J. C. Rodrigues. (B) Image from T. Souto-Pradrón. (C) Image reproduced with permission from Ref. [58] © (2009) Elsevier.

peptides and others. Although many studies have been published on *T. cruzi*, *T. brucei* and *Leishmania*, few have characterized the effects of these products on the ultrastructure of the parasites. Mitochondrial swelling and the presence of concentric membrane structures in its matrix were observed after incubation of *Leishmania* and *T. cruzi* with any of the following: linalool-rich essential oil of *Croton cajucara* [69], usnic acid from the lichen *Cladonia substellata* [70], extract of the marine sponge *Plakortis angulospiculatus* [71], extract of the seeds of *Pterodon pubescens* with geranylgeraniol (GG-OH) being the most active fraction [72]), propolis [73], taxol isolated from *Taxus brevifolia* [74], dinitroaniline herbicides [75], β -carboline [76], chalcones from *Piper aduncum* [77], or neolignan from leaves of *Piper regnellii* [78].

2.2. Kinetoplast

The kinetoplast is a fibrous network of the mitochondrial DNA (kDNA) of the protozoa Trypanosomatidae and Bodonidae. The kDNA, which represents about 30% of the total cellular DNA, may be dispersed or concentrated in a specialized region of the mitochondrion near the basal body, from which the flagellum emerges [79]. The kinetoplast shape and its localization relative to the nucleus vary according to the developmental stage of the different trypanosomatids. Two main forms are observed in *T. cruzi*, *T. brucei* and *Leishmania*: a compact rod- or bar-shaped structure observed in epimastigotes, promastigotes and amastigotes and a round form with a more dispersed array of the kDNA filaments, which is found in trypomastigote forms. The kDNA is composed of two circular types of DNA, maxicircles (small in number), which encode rRNAs and mitochondrial proteins, and thousands of minicircles, which encode guide RNAs that modify the maxicircle transcripts in a process known as RNA editing. According to Motta, maxicircles and minicircles are connected in a special catenated network array that resembles the chain mail of medieval armor [80]. Kinetoplast DNA minicircles have extensive closely-spaced phased

AT sequences that yield curved double-helical structures and provide potential cellular targets for diamidines that have AT sequence binding specificity [81].

The main alteration observed in the kinetoplast as an effect of the different drugs cited above is the disorganization of the kDNA network. There are different grades of disorganization such as: a) looser organization of the DNA filaments in the kDNA network; b) fragmentation of the kDNA, forming many clusters of electron-dense structures inside the mitochondrion; and c) compaction and multiple kDNAs.

Looser kDNA organization is observed when parasites are incubated with naphthoquinones and naphthoimidazoles [55-58] (Fig. 2E). Sometimes, the disorganization of the kDNA network, which has mainly been observed in trypomastigotes, seems to be the result of the severe swelling of the mitochondrion and the loss of the inner membrane. In contrast, aromatic diamidines [18, 20-23], natural and synthetic β -carboline [76], topoisomerase inhibitors [80, 82, 83], and DAB [68] cause a drastic effect: the destruction or fragmentation of the kDNA network in promastigotes and amastigotes of *Leishmania* and in trypomastigotes of *T. cruzi*. These effects were not observed in the kinetoplasts of epimastigote forms of *T. cruzi*. It is important to note that the looser kDNA aspect observed in pathogenic trypanosomatids after chemotherapy is not similar to the constitutive looser arrangement observed in endosymbiont-bearing trypanosomatids, such as *Blastocrithidia culicis*, *Crithidia deanei* and *Bodo* spp. [80].

Compaction of the kDNA filaments and the presence of multiple kinetoplasts were obtained after treatment of *T. cruzi* epimastigotes with piperine, which is an alkaloid of the *Piper* species. In some parasites, more than one individualized kinetoplast can be observed, mimicking the effects of cytoskeleton inhibitors. In other parasites, however, up to three kDNA bars can be seen inside the same mitochondrion [84] (Fig. 2J).

No structural changes in the kDNA network of *T. cruzi* or *Leishmania* were described in the presence of LPA analogues or SBIs [37, 39-42, 44-46, 50, 52].

2.3. Nucleus

The trypanosomatid nucleus presents a typical nuclear membrane with pores, condensed chromatin of varied density dispersed throughout the nuclear matrix up to the nuclear periphery. The nuclear envelope remains intact during the entire division process, with the appearance of intranuclear microtubules, chromatin dispersion and initiated formation of dense plates, the number of which varies according to the trypanosomatid species [8].

Some ultrastructural effects observed in the nucleus in the presence of different drugs indicate which death pathway the protozoan is undergoing. Different compounds may induce different types of programmed cell death (PCD), including apoptosis (type I PCD), autophagic cell death (type II PCD) and programmed necrosis (type III PCD) [10, 57, 58].

Aromatic diamidines and related analogues are DNA minor groove-binding ligands at AT sequences, and they exhibit strong antiparasitic activity [17]. Furamidine (DB75), its *N*-phenyl-substituted analogue (DB569) [18], and reversed [22, 23] and aromatic diamidines [26, 85] induce severe alterations in nuclear morphology. After DB75 and DB569 treatment, *T. cruzi* trypomastigotes presented characteristics of type I PCD [20, 21].

Treatment of *L. chagasi* promastigotes with ER-119884, an SQS inhibitor, resulted in altered nuclear chromatin organization with some alterations in the nuclear envelope [42].

One of the most remarkable ultrastructural effects observed in *T. cruzi* treated with naphthofuranquinones is strong nuclear envelope swelling [57, 58]. Similar effects were seen in *L. chagasi* promastigotes treated with telocinobufagin, a steroid isolated from *Rhinella jimi* parotid macrogland secretions [86], and in *T. cruzi* forms treated with *Ocimum basilicum* essential oil [87] or L-leucine methyl ester (Leu-OMe) [88], where the condensed nuclear material became separated from the nuclear membrane with leakage of its content.

Treatment with different DNA topoisomerase inhibitors causes morphological alterations to the parasites' nuclei. Characteristics like nuclear envelope distension and chromatin condensation were observed in *T. cruzi* epimastigotes after incubation with bacterial DNA topoisomerase type II [89]. Recently, the use of aspartyl peptidase inhibitors (PIs) has been demonstrated to exert a direct effect on *Leishmania* [90, 91]. *L. amazonensis* promastigotes treated with lopinavir, an HIV PI, presented condensed chromatin close to the nuclear envelope, a feature suggestive of apoptotic death [91].

T. cruzi trypomastigotes treated with suramin exhibited an altered nuclear division pattern and frequently presented irregular or multiple nuclei [92]. Vinblastin-resistant *L. amazonensis* also presented dysfunctional cytokinesis, in which the resistant promastigotes showed multiple nuclei [93]. This characteristic was found in parasites treated with the squalene synthase inhibitors BPQ-OH, ER-119884 and E5700;

promastigotes were frequently multinucleate (Fig. 2F) with unusual chromatin condensation [40, 41]. *L. amazonensis* amastigotes treated with edelfosine also presented abnormal numbers of nuclei [50].

2.4. The Cell Surface

The cell surface of trypanosomatids, as observed by TEM, is composed of three different components: the cell coat (or glycocalyx), the cell membrane and the sub-pellicular microtubule layer. The cell coat is in direct contact with the extracellular medium and is composed of oligosaccharides associated with membrane lipids and proteins. The use of freeze-fracture and freeze-etching with TEM has shown that the cell membrane of trypanosomatids presents domains characterized not only by a distinct chemical composition, but also by association with the sub-pellicular microtubules. The first and largest domain is composed of the membrane lining the cell body. The second and the third are the flagellar membrane and the flagellar pocket membrane, respectively [94].

Different drugs have different effects on the cell body and the flagellar membranes (Figs. 2I). Aromatic diamidines [26] and reversed diamidines [22, 23] cause detachment of the plasma membrane in areas where sub-pellicular microtubules are not observed. The presence of blebs budding from the cell body membrane and from the flagellar membranes are observed in epimastigotes of *T. cruzi* and promastigotes of *Leishmania amazonensis* after treatment with SMT [39, 46] and SQS inhibitors [41], respectively. Rupture of the flagellar membrane was also observed in promastigotes of *Leishmania chagasi* incubated in the presence of BPQ-OH, an SQS inhibitor [42].

Synthetic naphthoquinones caused the formation of blebs in the plasma and flagellar membranes of *T. cruzi* epimastigotes [58]. Naphthoimidazoles derived from β -lapachone showed a preferential effect on trypomastigotes in which bleb formation was observed throughout the cell body and flagellar membranes. Blebs associated with the cell body membrane did not present any sub-pellicular microtubules [57].

Lysophospholipid analogues caused the formation of blebs in *L. amazonensis* and *T. cruzi*, and the effects were more evident when edelfosine, miltefosine and ilmofosine were combined with ketoconazole [50-52] (Fig. 2F).

Plasma membrane disruption was described after treatment of *T. cruzi* and *T. brucei* with antimicrobial peptides such as defensins and cathelicidins, respectively [95, 96], and in *Leishmania* after incubation with cecropin A-melittin peptides and with the essential oil of *Croton cajucara* [69, 97].

The last kind of cell surface change observed after treatment of trypanosomatids with different drugs is alterations in the amount and/or localization of surface molecules. Do-campo *et al.* showed that treatment of *T. cruzi* with miconazole and econazole caused enhanced response of the parasite to the lectins WGA (wheat germ agglutinin) and PHA (phytohemagglutinin) [47]. Treatment of *T. cruzi* with suramin, a symmetrical polysulfonated derivative of urea used in the prophylactic treatment of human trypanosomiasis in Africa, caused increased activity of a Mg^{2+} -ecto-ATPase and the

FRA antigen and redistribution of negative surface charges. Alterations in extracellular components in the region of adhesion between the cell body and the flagellum and an increase in the adhesion of epimastigotes to resident macrophages were also observed [92, 98, 99] (Fig. 2L).

2.5. Cytoskeleton

Trypanosomatids present a continuous layer of microtubules immediately below the cell membrane, named the sub-pellicular microtubule layer. They are distributed throughout the protozoan body, with the exception of the flagellar pocket. The number of microtubules varies in different regions of the body of the parasite, but they are regularly spaced and connected by filaments to each other, to the inner face of the plasma membrane and to cytoplasmic organelles, such as the endoplasmic reticulum. One of the characteristics of the sub-pellicular microtubules is their high proportion of acetylated and/or polyglutamylated tubulins, which make them very stable [100]. Although actin and actin-binding proteins have been characterized in trypanosomatids, in *T. cruzi* they do not seem to be associated with sub-pellicular microtubules, as observed in *Leishmania* [101].

No damage to sub-pellicular microtubules was observed after treatment of *T. cruzi* and *Leishmania* with taxol, colchicine, vinblastine, trifluralin and ansamitocin P3, piperine or suramin. However, changes in the shape of the parasites, the presence of multiple flagellae, aberrant cell types with multiple kinetoplasts and absence of a nucleus were observed; these changes are probably due to incomplete differentiation and cytokinesis [74, 75, 84, 92, 102, 103]. Disorganization of the sub-pellicular microtubule layer was only observed in amastigotes of *T. cruzi* incubated in the presence of reversed amidines [22, 23].

2.6. Endoplasmic Reticulum and Golgi Complex

The endoplasmic reticulum (ER) and the Golgi complex (GC) in trypanosomatids have the same basic structure as that observed in all eukaryotic cells. The localization of the GC may vary according to the developmental form analyzed, but in all cases the CG remains near the flagellar pocket. Different classes of drugs affect the physiology of the ER-Golgi system, resulting in ultrastructural changes.

Sterol biosynthesis inhibitors have pronounced effects on the ER and GC [41]. Treatment with terbinafine and different azole derivatives caused modifications in ER-GC structures, which were characterized by the presence of large multivesicular bodies and autophagic structures [37, 38]. The inhibition of squalene synthase (SQS) by different inhibitors, such as BPQ-OH, ER-119884 and E5700, caused disorganization and fragmentation of Golgi complex cisternae, the appearance of myelin-like figures in the cytoplasm and an increase in the number of autophagosomal structures and multivesicular bodies characteristic of autophagy [39-42] (Fig. 2G).

Another class of enzyme inhibitor that has been extensively tested against *T. cruzi* is the cysteine protease inhibitors (CPI) [104-106]. They showed remarkable effects on GC structure in *T. cruzi* epimastigotes and intracellular amastigotes, which presented an increase in size and in the

number of cisternae (from 5-7 to up to 16) in addition to the presence of dilated vesicles.

Epimastigotes and trypomastigotes treated with protein kinase (PK) and phosphatidylinositol-3 kinase inhibitors, such as genistein, staurosporine and wortmannin, also presented autophagosomal structures that were characterized by ER profiles surrounding several organelles [107].

Swelling of the ER-Golgi system is a common effect in *T. cruzi* trypomastigotes treated with reversed amidines [22, 23] and DAB [66]. Vinblastine treatment also caused *Leishmania* ER cisternae to protrude outward and to contact the plasma membrane [93].

Natural products like plant-derived drugs, essential oils and animal venoms have remarkable effects on protozoa. Proanthocyanidin (an ethanolic extract from the *Kola acuminata* plant) seems to drastically increase the *T. brucei* rough ER content and the Golgi apparatus, which present up to seven to eight cristae [108]. In addition to inducing ER profiles surrounding cytoplasmic membrane structures, β -lapachone derivatives also induced remarkable Golgi alterations, like disruption and enlargement of the *trans*-Golgi network cisternae [55, 56]. Similarly, GG-OH treatment also caused effects on *T. cruzi* such as ER profiles surrounding organelles and myelin-like figures [72], suggesting an autophagic death pathway in response to both compounds. Dilation of the Golgi complex cisternae is also one of the effects of Brazilian green propolis on *T. cruzi* epimastigotes [109].

2.7. Endocytic/ Exocytic Related Organelles

The survival of trypanosomatids in distinct environments requires exogenous molecules that are internalized by fluid-phase and receptor-mediated endocytosis [110-113]. The endocytic pathway in trypanosomatids presents a singular architecture, in which the intracellular itinerary and delivery of internalized molecules vary significantly according to the developmental form analyzed [114, 115]. In trypomastigote forms of *T. brucei*, the endocytic/exocytic apparatus is restricted to the posterior region of the cell between the flagellar pocket and the nucleus. However, in epimastigotes of *T. cruzi* and promastigotes of *Leishmania*, molecules that enter the cytostome or are internalized from the flagellar pocket pass through a network of tubules and vesicles extending from the anterior to the posterior end of the parasite [112, 116-118]. The nature and the morphological aspect of the compartments of the endocytic/exocytic pathway in trypanosomatids vary according to the species analyzed. In African trypanosomes, the compartments are similar to those described in mammals, including the presence of lysosomes. *Leishmania* and *T. cruzi*, however, present different compartments that are potential drug targets.

Aromatic diamidines, such as pentamidine, caused remarkable changes in the flagellar pocket of amastigotes from *L. tropica* [12] and trypomastigotes of *T. rhodesiense* [119]. The flagellar pockets were frequently dilated and filled with double membrane-bound bodies that budded from the flagellar pocket membrane. The same phenomenon was also observed in promastigotes of *L. amazonensis* after incubation in the presence of azasterol and squalene synthase inhibitors, suggesting alterations in the secretory pathway [41, 44].

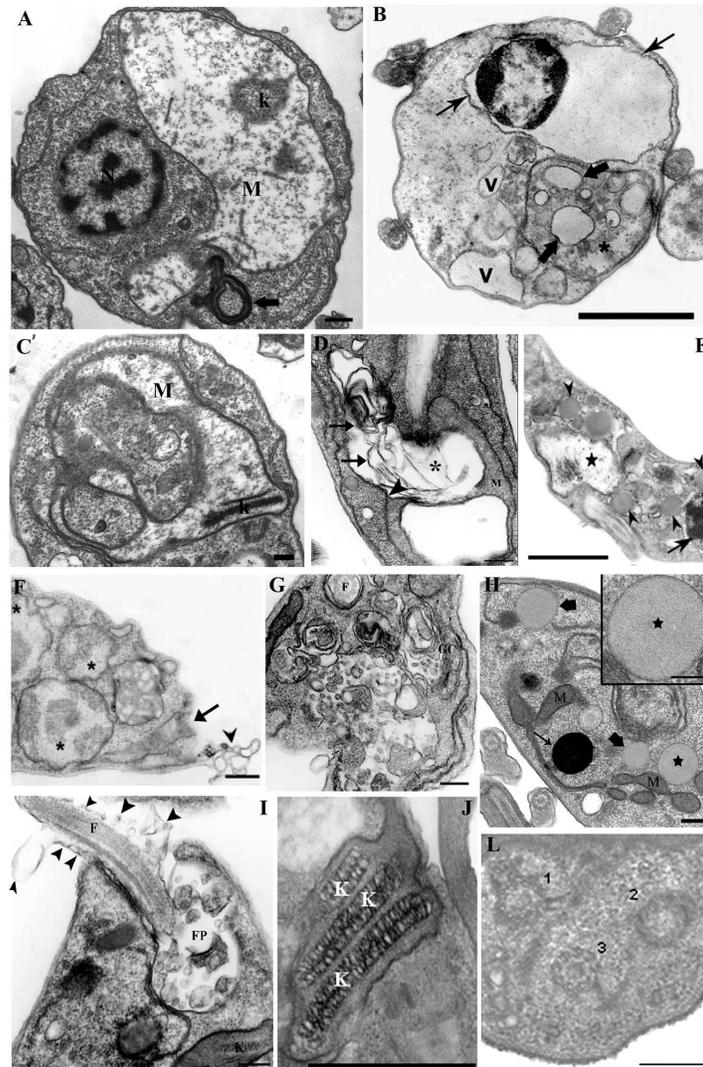


Fig. (2). Morphological alterations of parasites after different drug treatments. (A) An *L. amazonensis* promastigote treated with an SMT inhibitor for 96 h displaying a swollen mitochondrion and kinetoplast, altered nuclear chromatin distribution and presence of RE profiles (thick arrow). (B) Trypomastigote forms treated with naphthofuranquinone presenting mitochondrial swelling with intra-mitochondrial vesicle formation (thick arrows), increased cytoplasmic vacuoles (V), and strong nuclear envelope dilation (thin arrows). (C) *L. amazonensis* promastigotes showing intense mitochondrion swelling after $\Delta^{24(25)}$ sterol methenyl transferase inhibitor incubation. (D) *L. amazonensis* promastigotes forms presenting altered mitochondria with intense swelling and a less electron-dense matrix (arrows), alterations in the mitochondrial membranes such as the separation of the inner and outer membranes (arrowheads), and the presence of some concentric membranes and myelin figures in the matrix (arrows). (E) Naphthoimidazole (derived from β -lapachone) treatment leads to kDNA disorganization, swelling of the mitochondrion (asterisk), a decrease in the electron density of acidocalcisomes (arrowheads) and abnormal chromatin condensation (arrow). (F) Epimastigotes treated with edelfosine plus ketoconazole display alterations in the mitochondrion (arrow), plasma membrane (arrowheads), and appearance of multinucleated parasites (asterisks). (G) *L. amazonensis* promastigotes treated with 3 μ M BPQ-OH for 24 h display large vacuole-containing vesicles with a part of the cytoplasm, and some alterations in the Golgi complex (arrowheads). (H) *L. amazonensis* promastigotes treated with an SQS inhibitor showing the presence of many lipid droplets with distinct morphologies in the cytoplasm, a population of lipid bodies surrounded by a monolayer (thick arrows and star), and the presence of increased numbers of electron-dense inclusions, confirming the alteration in the sterol content. (I) *L. amazonensis* promastigotes showing several alterations in the flagellar membrane (arrowheads) after BPQ-OH treatment. (J) Some epimastigotes treated with the alkaloid piperine display triple k-DNA without complete division of the parasite. (L) Suramin treatment results in differentiation of trypomastigotes, which present up to three basal bodies. N, nucleus; K, kinetoplast; M, mitochondrion; F, flagellum. Bars: A, C: 6 μ m; B, D, E: 2 μ m; F: 0.25 μ m; G: 0.15 μ m; H: 0.5 μ m; J, I: 1 μ m; L: 0.3 μ m. (A) Courtesy of J.C. Rodrigues; (B) Image reproduced with permission from Ref. [58] © (2009) Elsevier; (C) Courtesy of J.C. Rodrigues. (D) Image reproduced with permission from Ref. [40] © (2005) Elsevier; (E) Image reproduced with permission from Ref. [55] © (2005) Oxford University Press; (F) Image reproduced with permission from Ref. [51] © (2005) Oxford University Press; (G) Image reproduced with permission from Ref. [40] © (2005) Elsevier; (H) Image reproduced with permission from Ref. [41] © (2007) American Society for Microbiology; (I) Image reproduced with permission from Ref. [40] © (2005); (J) Image reproduced with permission from Ref. [84] © (2008) Springer-Verlag; (L) Image reproduced with permission from Ref. [92] © (2008) Elsevier.

The lysophospholipid analogues (LPAs) edelfosine, miltefosine and ilmofosine, in addition to inducing the formation of vesicles and myelin-like figures in the flagellar pocket of *L. amazonensis* promastigotes [50], also induce morphological alterations in the reservosomes in epimastigotes of *T. cruzi*, which are characterized by swelling and the concentration of several reservosomes near the nucleus [51]. Reservosomes are also the main targets of cysteine protease antagonists. After incubation of epimastigotes in the presence of Z-(SBz)Cys-Phe-CHN₂, an irreversible cysteine-protease inhibitor, reservosomes became enlarged and more electron-dense [120].

Treatment with β -lapachone derivatives, anti-microtubule agents, risedronate or propolis induced various alterations in reservosome ultrastructure characterized by marked alterations in their morphology and in their electron density, due to inhibition of endocytic activity and/or to the increased degradation of the protein matrix. The presence of electron-lucent rod-shaped inclusions was observed [56, 73, 74, 103, 109, 121].

Active plant components also interfere with compartments of the endocytic pathway in trypanosomatids. The proanthocyanidin from *Kola acuminata* caused enlargement of the flagellar pocket and of the lysosome-like structures as well as the formation of multivesicular bodies in bloodstream *T. brucei* [108]. Flagellar pocket swelling was observed in *L. chagasi* promastigotes treated with *Cymbopogon citratus* essential oil [122].

2.8. Acidocalcisomes

The acidocalcisome is an acidic electron-dense organelle enclosed by a single membrane that is found in all members of the Trypanosomatidae family. The number and size of acidocalcisomes in a cell varies, and they can be observed in all developmental forms of *T. cruzi*, *T. brucei* and *Leishmania*. The electron-dense matrix contains sodium, magnesium, potassium, calcium, zinc, iron and phosphorous in the form of inorganic pyrophosphate and polyphosphate, as determined by electron microscopy microanalysis and energy-filtered transmission electron microscopy. In addition to storing the ions listed above, acidocalcisomes are also involved in pH homeostasis and osmoregulation. In some protozoan parasites (including trypanosomatids), the acidocalcisomes present enzymes that are not found or different from those found in mammals, such as the vacuolar proton translocating pyrophosphatase (V-H⁺-PPase) and a soluble inorganic pyrophosphatase (PPase) [123,124]. They are inhibited by various bisphosphonate pyrophosphate analogues, some of which are commercially available for the treatment of bone resorption diseases.

Aromatic diamidines, such as furamidine and the other fluorescent compounds suramine and chloroquine, were observed to accumulate in the acidocalcisomes of *T. brucei* [30, 31, 125]. Naphthoimidazole N1, a β -lapachone derivative, caused a decrease in the electron density of acidocalcisomes in *T. cruzi* trypomastigotes [55] (Fig. 2E). The most evident effect on the ultrastructure of acidocalcisomes was described in *Leishmania amazonensis* after treatment with the SBIs ketoconazole and terbinafine. The authors described a change in electron density of the cytoplasm, an increase in

volume and in the number of the organelles, and the presence of acidocalcisome cores inside large vacuoles presenting characteristic autophagic-like structures. This last feature was also observed after incubation of *Leishmania* with the SBI 22, 26-azasterol [44]. An increase in acidocalcisome volume was also observed after incubation of *L. chagasi* promastigotes in the presence of essential oil from *C. citratus*, *Lippia sidoides*, and *Ocimum gratissimum* [122].

2.9. Glycosomes

Glycosomes are spherical or elongated structures with a homogeneous matrix surrounded by a single membrane. Most of the glycolytic pathway of trypanosomatids takes place in this organelle [126]. Besides glycolytic activity, which in trypanosomatids plays an essential role in their ATP supply, other metabolic pathways have been described to occur in glycosomes, such as isoprenoid and sterol biosynthesis. The enzymes in the glycosomes that participate in the different metabolic pathways are structurally and kinetically different from those observed in mammalian hosts, making the glycosomes a promising target for new drugs against parasitic trypanosomatid protozoa. Glycosomes in monoxenous trypanosomatids also contain catalase and are thus considered a special type of peroxisome.

Ultrastructural changes in glycosomes have been described only after incubation in the presence of the SB inhibitor terbinafine, which caused an increase in the number of glycosomes in epimastigotes of *T. cruzi* [37]. The authors also observed glycosomes arranged in stacks after 144 h of treatment.

FINAL REMARKS

Cell biology studies using electron microscopy have made important contributions to the identification of main targets for different drugs against trypanosomatids and to revealing their effects at the cellular level. New synthetic and natural compounds used in nanomolar concentrations and combinations of new and old drugs have been successfully used. In addition to the most commonly observed effects, which are mitochondrial swelling and cytoplasm vacuolization, some drugs cause a complex sequence of ultrastructural damage, indicating the progression of apoptosis-like and autophagic cell death in *Leishmania* and *T. cruzi* [127, 128].

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