Electron Microscopy in Antiparasitic Chemotherapy: A (Close) View to a Kill

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Abstract: Electron microscopy may be useful in chemotherapy studies at distinct levels, such as the identification of subcellular targets in the parasites and the elucidation of the ultimate drug mechanism of action, inferred by the alterations induced by antiparasitic compounds. In this review we present data obtained by electron microscopy approaches of different parasitic protozoa, such as *Trypanosoma cruzi*, *Leishmania* spp., *Giardia lamblia* and trichomonads, under the action of drugs, demonstrating that the cell architecture organization is only determined in detail at the ultrastructural level. The transmission electron microscopy may shed light (*i.e.* electrons) not only on the affected compartment, but also on the manner it is altered, which may indicate presumable target metabolic pathways as well as the actual toxic or lethal effects of a drug. Cytochemical and analytical techniques can provide valuable information on the composition of the altered cell compartment, permitting the *bona fide* identification of the drug target and a detailed understanding of the mechanism underneath its effect. Scanning electron microscopy permits the recognition of the drug-induced alterations on parasite surface and topography. Such observations may reveal cytokinetic dysfunctions or membrane lesions not detected by other approaches. In this context, electron microscopy techniques comprise valuable tools in chemotherapy studies.

Key Words: Electron microscopy, chemotherapy, pathogenic protozoa, drug targets.

INTRODUCTION

Electron microscopy has usually played a pivotal role in protistology and cell biology, as well as on histology and pathology. Contrary to the high-tech appealing of molecular biology, genomes, transcriptomes and proteomes, electron microscopy remains largely dependent on hand skills and sharps-eyed, long-trained researchers [1]. The diversified and prolonged training required for the proper employment of ultrastructural techniques led to a reduced number of studies focusing on both molecular biology and cellular structure aspects.

The neglected tropical diseases are a group of 13 infections that affect at least 2.7 billion people worldwide [2]. Seven of them are caused by worms, three are bacterial and the other three are caused by pathogenic trypanosomatids (African trypanosomiasis, Chagas disease and leishmaniasis). Later on, the parasitism by the protozoa *Giardia* and *Cryptosporidium* joined the Neglected Diseases Initiative [3]. However less than 1% of new drugs introduced in our therapeutical arsenal over the last 30 years has been directed to tropical diseases [4, 5]. There are no effective vaccines for such parasitic diseases and the current treatment still has important drawbacks such as variable efficacy, important side effects and development of drug resistance. Despite the prolonged and widespread use of many of such drugs, their unequivocal mechanisms of action, particularly on the parasite cellular organization, remain scarcely understood. Electron microscopy studies shed light on the cell biology of parasitic protozoa, including the biological roles of different organelles on the organism life cycle and their participation on the outcome of chemotherapy [6-8].

In the present review we focus on the role of electron microscopy approaches in determining subcellular drugs targets and elucidating the mechanisms of action of antiparasitic compounds.

SUBCELLULAR TARGETS

Cell biology approaches may shed light on antiprotozoal chemotherapy [6, 9]. The observation of ultrastructural alterations in drug-treated parasites may reveal the primary and secondary target sites within the pathogens. According to the compartment(s) affected, different enzyme sets or metabolic pathways may be implicated as targets. The manner in which these compartments are affected may be helpful in the elucidation of drug mechanism(s) of action at the cellular and subcellular levels.

CELL SURFACE

Parasite plasma membranes often display altered ultrastructure in response to microbicidal drugs. Parasites treated with microbicidal compounds may present morphological changes (Fig. (**1**)), assessed by scanning electron microscopy (SEM).

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Fig. (1). Drug induced surface alterations detected through scanning electron microscopy (SEM). Morphological alterations of *T. cruzi* trypomastigotes (**a, b**) and epimastigotes (**c, d**). Untreated parasites (**a, c**) display usual morphology and surface topology. Trypomastigote treated with the β-lapachone derivative N1, presenting a contorted cell body (**b**, arrowhead). Epimastigote treated with the β-lapachone derivative N3, displaying pronounced shrinkage of the cell body (**d**). Reproduced with permission.

Cell surface shrinkage, DNA fragmentation, loss of mitochondrial membrane potential and release of cytochrome suggestive of an apoptosis-like process were observed in *L. donovani* promastigotes treated with the lysophospholipid analogue (LPA) miltefosine [10, 11]. Such alterations were also observed in *T. cruzi* treated with thre naphthoimidazoles derived from β-lapa-chone, named N1, N2 and N3 [12, 13].

Surface shrinkage may indicate cytoplasmic loss as well as cytoskeleton disorganization. Multiple surfaces in folds may be brought by truncated or slowed cytokinesis. Sequenced attempts to accomplish cell division may lead to several furrows interrupting the protozoan outer cytoplasm (Fig. (**2**)). Surface-exposed structures such as flagella (Fig. (**2**)) undulating membranes and the *Giardia lamblia* adhesion disks (Fig. (**3**)) [14] may be altered in number and/or appearance in drug-treated parasites. Cytoplasmic organelle multiplicity may also indicate impaired cell division (*vide infra*). Cell surface discontinuities detectable by scanning microscopy may be indicative of membrane damage by factors such as oxidative stress, induced either by drugs or by immune response effector mechanisms.

Trypanosomatids present rather stable cell surfaces due to the steadiness of the subpellicular microtubules arrayed underneath the parasite plasma membrane [9]. It is possible to observe destroyed parasites, presumably by necrosis, still exhibiting well-preserved plasma membranes and associated microtubules. Although this microfilament connection seems

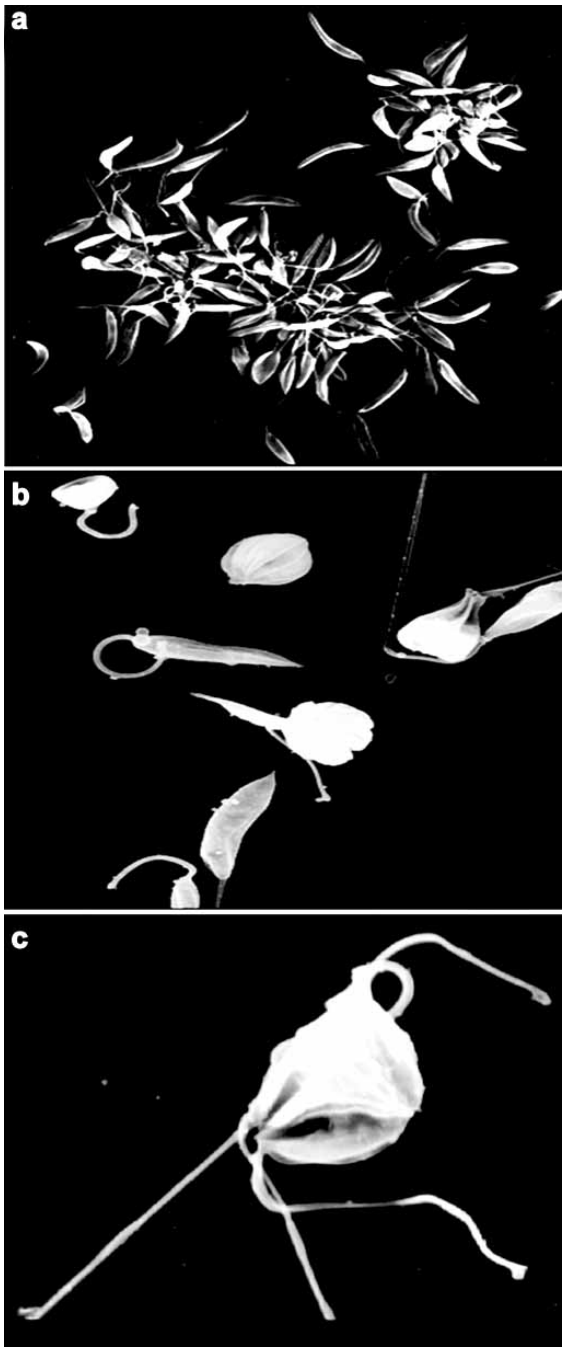

Fig. (2). *L. amazonensis* selected under vinblastine pressure displays MDR phenotype and considerable surface alterations. Contrary to control promastigotes (**a**), the MDR^+ parasites were bizarrely shaped cells (**b**) with short curved flagella or multiflagellate (**c**). Reproduced with permission.

significantly steady, the treatment with compounds able to disorganize the cytoskeleton such as the protein kinase C activator 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA, also known as phorbol myristate acetate) may lead to the forma-

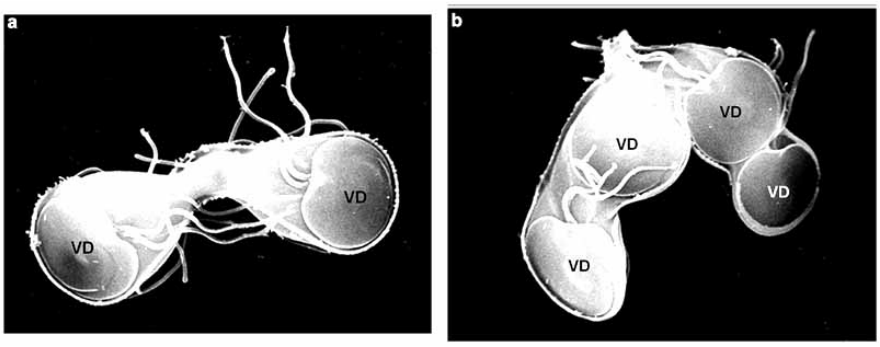

Fig. (3). The cell division in untreated *Giardia lamblia* is usually rather symmetrical (**a**) and may be truncated in trophozoites incubated with the putrescine analogue 1,4-diamino-2-butanone (DAB), which display multiple ventral disks (VD) and increased number of flagella. Reproduced with permission.

tion of filopod-like membrane protrusions (Fig. (**4**)) in *Leishmania amazonensis* [15] and in *Trypanosoma cruzi* [16]. Such protrusions are usually devoid of cytoskeleton elements and the microtubules remain regularly arrayed, but membrane protein distribution, assessed by freeze-fracture, may be disordered. Therefore membrane ruffles may indicate altered cytoskeleton organization and/or impaired plasmalemma-cytoskeleton connection. It must be considered that these parasites shed membrane fragments constitutively [17-19]. Plasma membrane ruffling and blebbing were also reported after treatment of different microorganisms with drugs. In *T. cruzi* the LPAs edelfosine, miltefosine and ilmofosine induced blebbing and ruffling of the membrane and such alterations were associated to interference in phospholipid content [20, 21]. LPAs are also active on tumor cells [22], and cause changes in the membrane fluidity leading to its physical disruption [23, 24]. *L. amazonensis* promastigotes cultured with ketoconazole and terbinafine also display surface blebbing [25] (Fig. (**4**)). These and other compounds are intensively studied aiming new drugs for Chagas disease and leishmaniasis [26] and interesting results have been obtained targeting ergosterol biosynthesis. *Tritrichomonas foetus* treated with griseofulvin also produces membrane projections [27].

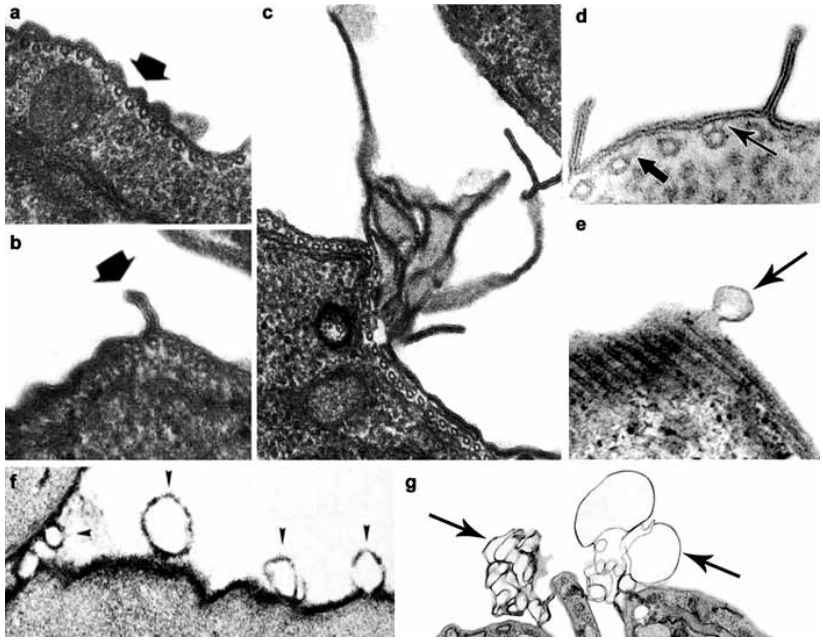

Fig. (4). Drug-induced cell surface projections observed under transmission electron microscopy (TEM). Phorbol ester-treated *L. amazonensis* promastigotes protrude membrane units devoid of cytoplasmic core (**a, b**), which eventually give rise to large membrane ruffles (**c**). Promastigotes cultured with vinblastine project fillopod-like structures (**d**). Note that the filaments connecting subpellicular microtubules to the plasma membrane (thin arrow) were eventually altered (thick arrow). Membrane blebbing (arrow) in *T. cruzi* trypomastigote treated with the β-lapachone derivative N2 (**e**). Acid phosphatase cytochemical detection on surface blebs induced by ketoconazole and terbinafine on *L. amazonensis* promastigotes (**f**). Plasma membrane shedding in edelfosine-treated *T. cruzi* epimastigote (**g**). Reproduced with permission.

Cytoskeleton-affecting drugs can induce the formation of membrane protrusions resembling surface blebs which may progress to membrane shedding [17, 19] or microparticle liberation. Small protrusions, devoid of cytoplasmic core, may be the outcome of diverse stimuli, activating the death-associated protein kinase (DAPk), observed in different cell death pathways, including autophagy [28]. In this regard, mitochondrial swelling, plasma membrane blebbing and autophagosome-like structures may be detected simultaneously in drug-treated parasites, indicating that different cell death mechanisms are triggered concurrently [8].

Treatment of *T. cruzi* with the putrescine analogue 1,4-diamino-2-butanone (DAB) or the naphthoimidazole derivatives of β-lapachone N1, N2 and N2 [12, 13] may trigger the formation of cytoplasm-containing surface protrusions that resemble apoptotic bodies (Fig. (**5**)) and therefore may be indicative of apoptosis induction.

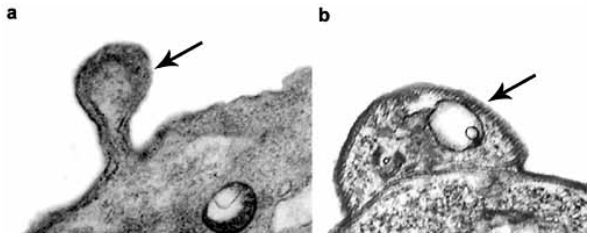

Fig. (5). Drug-induced *T. cruzi* surface protrusions. Epimastigote treated with DAB (arrow) (**a**) and trypomastigote, with the the β-lapachone derivative N1 (arrow) (**b**). Reproduced with permission.

CYTOSKELETON

Several drugs targeting the cytoskeleton, often developed for antitumoral purpose, were demonstrated to have antiparasitic activity [29, 30]. Colchicine, cytochalasins, taxol, nocodazole and griseofulvin were reported to produce remarkable ultrastructural alterations in the anaerobic protozoa trichomonads [31] and *Giardia* [32].

In *T. cruzi* epimastigotes taxol induced interruption of nuclear division, led to mitochondrial damage and to the appearance of multiflagellar forms with several kinetoplasts, while in trypomastigotes, this drug led to flagellar pocket dilatation [33, 34].

The dinitroaniline trifluralin is a microtubule-disrupting herbicide which has been investigated as antiproliferative agent on pathogenic protozoa [35]. Trifluralin inhibited promastigote proliferation of different species of *Leishmania* [36-38]. Trifluralin and its intermediate chloralin induced in *T. cruzi* the appearance of vacuoles containing damaged membranes, suggestive of an autophagic process, and for chloralin-treated parasites, blebs were also observed on the plasma and flagellar membranes. The treatment of epimastigotes with trifluralin induces mitochondrial swelling, an increase in the number of reservosomes, and occasionally parasites displaying three kinetoplasts were observed. Ultrastructural analysis of *T. cruzi* treated with taxol [34], trifluralin or chloralin [35] showing no damage in subpellicular microtubules does not exclude the possibility that these drugs interfere in their function. Microtubules in *T. cruzi* are very stable and much more resistant to colchicine and vinblastine than those of vertebrate cells [39], and this resistance was associated with a high content of acetylated and/or polyglutamylated tubulins [40].

Cytoskeleton-disturbing drugs may be used for selection of cells displaying the multidrug resistance phenotype (MDR). Multidrug-resistant *L. amazonensis* were obtained by *in vitro* selection with vinblastine and exhibit cross resistance to the chemically unrelated drug adriamycin [41]. Gueiros-Filho and coworkers [41] produced a vinblastine-resistant clone (CL2) and a cell line (RV100) presenting MDR phenotype. These parasite populations expressed a surface P-glycoprotein that pumps-out the drugs from the promastigote cytoplasm. Nevertheless drug resistance phenotype relies not only on drug extrusion. The MDR phenotype is multifactorial both in cancer cells [42] and protozoa [43]. Several mechanisms may be implicated, including cytoskeleton-mediated cytoplasmic distribution of the compounds. In this regard it is noteworthy that cytoskeleton elements such as the intermediate filaments cytokeratin and vimentin are required in the autophagy progression [44]. Multiflagellate and multinucleate cells often displaying multiple membrane in folds (Figs. (**2**) and (**3**)) presumably resulting from truncated or impaired cell division [14, 45]. Similarly, *Vinca* alkaloids produced a reversible blockage of cytokinesis in *T. cruzi* with the presence of multiple nuclei and kinetoplasts [46]. The occurrence of multiple basal bodies in vinblastin-cultured *Leishmania* [45] and suramin-treated *T. cruzi* [47] may be indicative of impaired cell division control.

MITOCHONDRIA

The mitochondrial ultrastructure is related to its function [48]. Numerous compounds were reported to affect mitochondria of different cell types as pharmacological targets, including mammalian [49-52] and parasitic species models [53-55]. Mitochondrial damage may trigger caspase-dependent programmed cell death (PCD) brought by cytochrome c release *via* mitochondrial permeability transition (MPT). Several antibiotics selectively affect both bacteria and mitochondria and medications may lead to mitochondrial damage [56, 57]. The endosymbiotic origins of the organelle may explain, at least in part, such selectivity of antibiotics such as ciprofloxacin, tetracycline, doxycycline, clindamycin and spiramycin against apicoplasts of apicomplexan parasites [58] and the metronidazole activation in hydrogenosomes of trichomomad parasites [59]. *T. cruzi* mitochondrion is the target site of the gentian violet [60] used in the prophylaxis of Chagas disease transmission *via* blood transfusion [61]. The mitochondrial targeting of drugs may rely on free radical production and/or on calcium homeostasis [62]. In intracellular *T. cruzi* amastigotes the calcium-modulating drug risedronate induced mitochondrial swelling and blocked the differentiation to trypomastigote, with no biochemical or ultrastructural effects on host cells, which could fully recover their normal structure and activity [63].

Ergosterol biosynthesis inhibitors (EBIs) produce mitochondrial damage on several parasitic protozoa. One of the characteristic ultrastructural effects of these inhibitors on trypanosomatids is a marked swelling of their single giant mitochondrion, correlated with the depletion of the endoge-

nous parasite sterols, which can lead to cell lysis [25, 64-68]. Epimastigotes of *T. cruzi* treated with ketoconazole plus the LPA edelfosine presented also severe mitochondrial swelling, with a decrease in electron density of its matrix and appearance of concentric membranar structures inside the organelle [69]. The group of Urbina has shown that *T. cruzi* mitochondrial membranes, in contrast to those of vertebrate cells, are indeed rich in specific parasite's sterols, which are probably required for their energy transducing activities [70].

Furthermore ergosterol may play a part in viscosity and in organelle stability; therefore ergosterol-deficient mitochondria may be more friable and prone to rupture (Fig. (**6**)). Similarly, polyamines incorporated to this organelle may participate in membrane stabilization. In this regard, putrescine, the diamine employed for polyamine synthesis, was shown to preserve mitochondrial function during subcellular fractionation [71] and its analogue DAB was demonstrated to cause mitochondrial destruction in *T. cruzi* and *L. amazonensis* (Fig. (**6**)) [72, 73]. In these cases the organelle is remarkably swollen and electrolucent.

The alteration in redox organelles may be brought by reactive oxygen species (ROS) enhanced production in polyamine-deprived parasites as these polycations are antioxidant and take part in the synthesis of trypanothione (N(1), N(8) - bis(glutathionyl)spermidine). Under conditions of oxidative stress or mitochondria malfunctioning, the organelle may be selectively degraded by mitoptosis or mitophagy. In ROS-induced mitoptosis, mitochondrial reticulum undergoes fission or thread-grain transition, a process required for mitoptosis in other cell types [74], but it is not know whether such transition takes place in the single mitochondrion of the trypanosomatid parasites. Different microscopy techniques demonstrate that malfunctioning mitochondria may be aggregated in the nuclear area, form membrane-bounded mitoptotic bodies and get decomposed and extruded from the cells [75].

Mitochondria can undergo destruction by autophagy (*vide infra*) or ubiquitination which may signal the organelle entry in multivesicular bodies (MVB). Nevertheless it should be remarked that it is simply not enough to report drug-induced mitochondrial damage. It is important to determine how it is affected to infer the underlying process taking place. Different EBIs produce mitochondrial damage, but the organelle may be affected in distinct forms. Ketoconazole and terbinafine dramatically affected the *L. amazonensis* single mitochondrion [25], but not in a simple way. Some organelles were reported to be disrupted, forming small vesicles (Fig. (**6**)) and other mitochondria were observed in fusion with MVB (*vide infra*) (Fig. (**10**)). These observations may indicate decreased membrane viscosity, and therefore fusogenicity. The former mechanism would result in leakage of mitochondrial contents to the cytoplasm, possibly including cytochrome c and apopotosis-inducing factor (AIF), and the latter one would lead to autophagic destruction of the organelle. The uncontrolled fusion with different compartments may cause discrete outcomes. The rise of circular membranes and lipid inclusions within mitochondria may be due to membrane fusion and lipid disorganization. Rather than static inner membrane invaginations, the mitochondrial cristae (Gr. crests) are dynamic structures with a topology influenced by factors such as ADP concentration and osmotic stress. Circular cristae formation is induced not only by polyamine or EBIs, but also by geranylgeraniol, propolis and the dinitroanilines trifluralin and chloralin [8], presumably among many others. Fusion of the cristae may have produced interior structure of the relict mitochondrion in the anaerobic protozoan *Cryptosporidium parvum* [76].

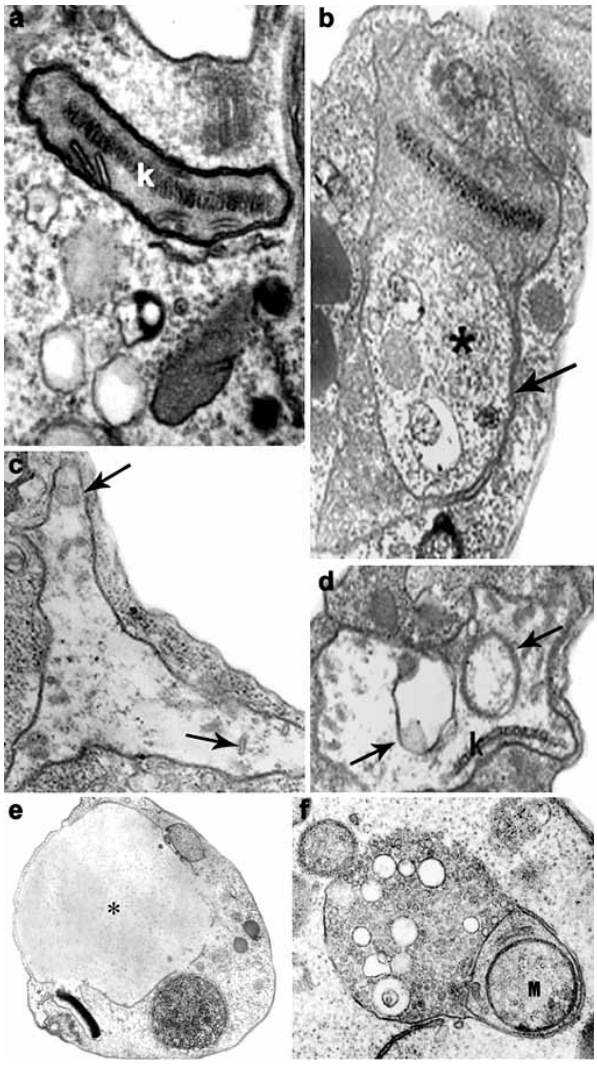

Fig. (6). Drugs targeting parasite mitochondria. Untreated *L. amazonensis* promastigote showing normal kinetoplast (k) and mitochondrion (**a**). Mitochondrial fenestration in DAB-treated promastigote (**b**). Note that the cytoplasm (*) may be observed through the organelle and that in a tangential section it may be mistaken for an endoplasmic reticulum cistern (arrow). DAB treatment ultimately leads to the complete destruction (**c, d**), which is often hardly identified except for scarce cristae (c, arrows) or kinetoplast DNA (K) (d). Circular cristae may be detected (d, arrows). *T. cruzi* epimastigote incubated with the dinitroaniline chloralin displaying remarkably swollen mitochondrion (*) (**e**). Mitochondrial (M) disruption in *L. amazonensis* promastigote cultured with ketoconazole and terbinafine (**f**). Reproduced with permission.

The topography of the mitochondrial inner membrane can remarkably influence its bioenergetic and death-triggering functions. Mitochondria structure may be regulated by a number of stimuli, including the immune response. Circular mitochondrial cristae may be formed in DNA-depleted fibroblasts [77], and in microglial cells treated with interferon-γ [78]. The dynamics of mitochondrial cristae topology may regulate PCD-type I (PCD-I) as the apoptosis-inducing cytochrome c is largely sequestered within the intracristae compartment and thus its transfer to the cytosol may be modulated by cristae fusion patterns [79]. It was shown that mitofilin, a homotypic assembling protein found in the space between the inner and outer mitochondrial membranes, controls mitochondrial cristae morphology [80].

Mitofilin down-regulation in HeLa cells using interfering RNA decreases proliferation and induces apoptosis. Interestingly these alterations are associated to increased reactive oxygen species ROS production. John and coworkers [80] reported that inner mitochondrial membrane remodeling may lead to formation of myelin figure-like structures which may well be mistaken for autophagic vacuoles (*vide infra*), made up of endoplasmic reticulum. The discrimination may be made by the presence of cytoplasmic contents such as ribosome remnants or the distinct distance between the organelle membranes, since the mitochondria membrane interspace is *circa* 23 nm wide, whereas the endoplasmic reticulum membranes are separated by 50-200 nm.

Mitochondria may also get fenestrated allowing the observation of the cytoplasm through the organelle (Fig. (**6**)). In this case, the tangential sections of the organelle may be mistaken for endoplasmic reticulum cisternae (Fig. (**6**)). In this regard the mitochondria of activated glial cells may be apparent as ring- or U-shaped slender cisternae [78].

Increased bilayer fluidity and so protein lateral motility may lead to the paracrystaline arrays observed in EBI-treated parasites (*vide infra*) (Fig. (**10**)). It should be kept in mind that mitochondrial longitudinal profiles display up to four membranes (not always visible in the section plane) and the mitochondrial matrix is considerably more electron dense than the endoplasmic reticulum lumen, mainly due to the iron content. Therefore a compound or combination of drugs may produce distinct effects targeting the same organelle, which may be deduced by electron microscopy.

KINETOPLAST

Kinetoplast is the enlarged DNA-containing region of the Kinetoplastida single mitochondrion. It lodges over 20% of the total parasite genome. It is comprised of two circular types of DNAs, maxicircles and minicircles [81]. Since the minicircles present high amounts of AT sequences, kDNA represents a potential drug target. Different drugs induce *T. cruzi* kinetoplast disorganization. Compounds such as DAB, geranylgeraniol, vinblastine produced mitochondrial swelling irregularly condensed and shapped kDNA (Fig. (**7**)).

In trypanosomatids, growing evidence supports that the kDNA is the primary target of aromatic diamidines [82-84]. Transmission electron microscopy (TEM) studies showed that the structure of parasite mitochondria (and kinetoplast) is highly altered by aromatic diamidines and reversed amidines, at doses that do not affect mammalian host cells [85, 86]. Flow cytometry studies confirmed that these compounds

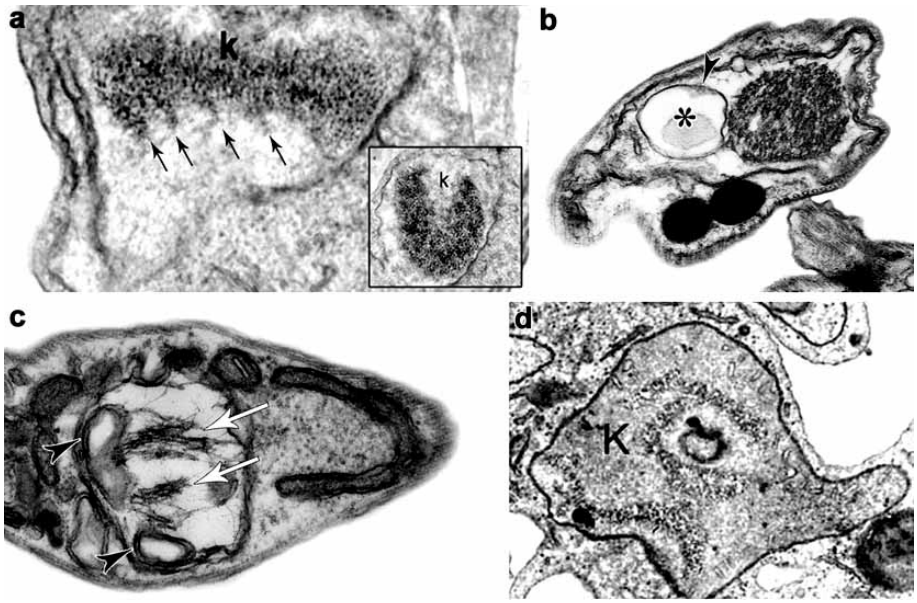

Fig. (7). Drug-induced kinetoplast disorganization. DAB-treated *T. cruzi* epimastigotes presented irregularly condensed and shapped (inset) kDNA (K) (**a**), presenting disordered fibrils (arrows). *T. cruzi* trypomastigotes treated with geranylgeraniol presented marked mitochondrial swelling (*) and kDNA alterations and circular cristae (arrowheads) (**b, c**). The compound led to a prominent kDNA disruption (c, arrows). *L. amazonensis* promastigotes cultured with vinblastine eventually presented enlarged mitochondria displaying multiple kinetoplast DNA (k) collections (**d**). Reproduced with permission.

target the mitochondria-kinetoplast complex of *T. cruzi* through the interference with the proton electrochemical potential gradient of the mitochondrial membrane [86, 87].

The kinetoplast may also be sensitive to drugs targeting the enzymes metabolizing mitochondrial genome like topoisomerases [88-90]. DNA topoisomerases II are enzymes that alter the topology of DNA and in kinetoplastids have been the focus of considerable study in the areas of molecular and cellular biology and also experimental chemotherapy. Several inhibitors of bacterial DNA topoisomerase II showed activity against *T. cruzi* inhibiting both proliferation and differentiation processes, and causing damage to the kinetoplast and the nucleus of epimastigotes [91, 92]. Camptothecin, inhibitor of eukaryotic DNA topoisomerase I, induced cleavage of nuclear and mitochondrial DNA in *T. cruzi* [93].

In studies about the effect of DNA topoisomerase I inhibitors on *L. donovani* it was reported that peganine hydrochloride dihydrate binds to the enzyme and induces loss of mitochondrial transmembrane potential and thus apoptosis in both stages of the parasite [94], and that 3,3'-diindolylmethane leads to ROS production by the mitochondrion through inhibition of F0F1-ATP synthase also leading to PCD-I [95]. Also, induction of apoptosis-like cell death by pentamidine and doxorubicin through differential inhibition of topoisomerase II was reported in arsenite-resistant *L. donovani* [96].

LYSOSOMAL AND ENDOSOMAL COMPARTMENTS

It is well established that the study of endocytic pathways may shed light on new drug delivery systems in different chemotherapic models [97, 98]. In addition, the parasite endocytic pathway may furnish promising targets for chemotherapy [99]. Nutrient uptake may be a useful drug target in *Plasmodium* sp. parasites [100].

L-aminoacyl methyl esters such as *L*-leucine methyl ester (Leu-OMe) have been identified as lysosomal system-targeting agents with antileishmanial properties, causing the lysis of amastigotes of *L. amazonensis* both isolated [101, 102] and within macrophage parasitophorous vacuoles [103]. These compounds affect *L. amazonensis* amastigote megasomes [104] and *T. cruzi* lysosomes [105]. Parasite lysis possibly occurred by a mechanism similar to that observed in mammalian lysosomes: *L*-amino acid methyl esters are entrapped by protonation, hydrolyzed by enzymes, and free amino acids do not diffuse out of the lysosome, due to their high polarity at acidic pH, resulting in water influx, swelling and disruption of the organelle [106].

The phospholipid analogue edelfosine, the bisphosphonate risedronate and the natural product propolis induced remarkable disorganisation and of *T. cruzi* epimastigotes reservosomes, which were distended and presenting reduced electron density (Fig. (**8**)) [63]. These drugs also led to the appearance of autophagic vesicles. The endocytic pathway may be the target of protease inhibitors such as the cysteine proteinase antagonist E-64, which may cause electron microscopy-detectable alterations in a *T. cruzi* cell line displaying resistance to cysteine proteinases synthetic inhibitor [107]. This parasite population seems to have evolved

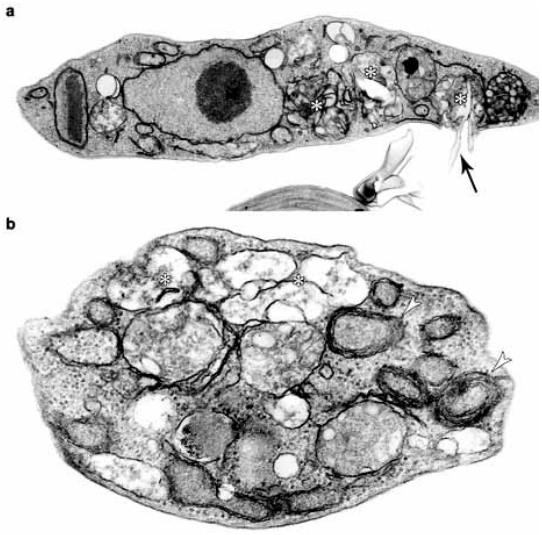

Fig. (8). *T. cruzi* endocytic pathway under drug effects. Epimastigotes treated with edelfosine (**a**) and propolis extract (**b**). In the parasites treated the phospholipid analogue membrane damage (arrow) was observed in association with reservosomes (*). The propolis-treated ones display numerous myelin-like figures (arrowheads). Reproduced with permission.

mechanism(s) to modulate endocytic compartment traffic. Enlarged electron dense reservosomes were also detected in *T. cruzi* epimastigote resistant to the irreversible cysteine proteinase antagonist Z-(SBz)Cys-Phe-CHN_2 (Fig. (**9**)), presenting enhanced expression of a 30 kDa cathepsin B-like cysteine protease [107], which was corroborated by immunogold cytochemistry.

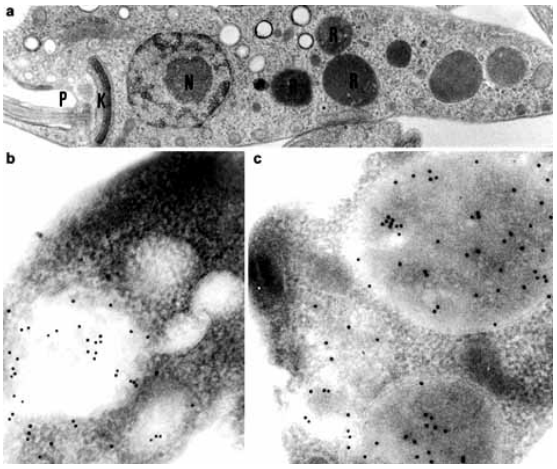

Fig. (9). *T. cruzi* epimastigote resistant to the irreversible cysteine proteinase inhibitor Z-(SBz)Cys-Phe-CHN_2, presenting enlarged electron dense reservosomes (R) at the posterior portion of the cytoplasm (**a**). Immunogold detection of the 30 kDa cathepsin B-like cysteine proteinases, using a monoclonal antibody on wild type (**b**) and resistant (**c**) epimastigotes. P, flagellar pocket; K, kinetoplast; N, nucleus. Reproduced with permission.

MVB are endosomal compartments found in numerous cell types [108] that may be involved in autophagic mitochondrial degradation [109]. EBI-treated *L. amazonensis* promastigotes and amastigotes presented, which were positive for acid phosphatase and formed at the trans-Golgi area. MVB fused with mitochondria [25], apparently in a microautophagy process (Fig. (**10**)).

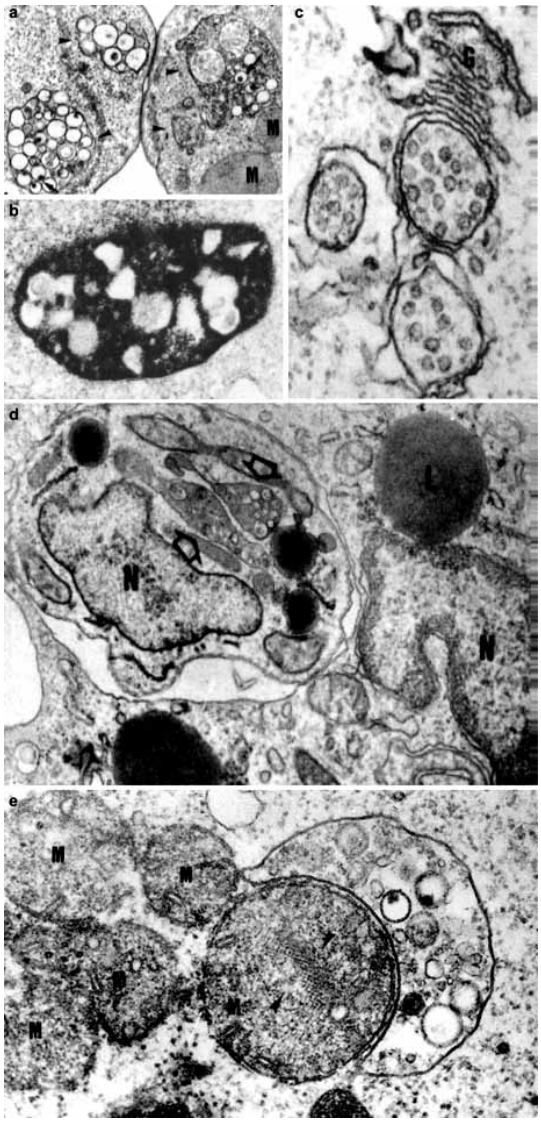

Fig. (10). Ketoconazole (a) or ketoconazole and terbinafine (b-e) induce multivesicular body formation in *L. amazonensis*. The ergosterol biosynthesis inhibitors induce multivesicular body (MVB) formation in promastigotes (**a**, arrowheads) as well as in intracellular amastigotes (**d**, arrows). These endosomal compartments were cytochemically positive for acid phosphatase (**b**) and were, presumably, formed at the trans-region of the Golgi (G) apparatus (**c**). Promastigote presenting fusion between multivesicular body (arrow) and mitochondrion (**e**, M). A paracrystaline protein array is observed within the mitochondrion (arrowheads). Reproduced with permission.

Parasite lysosomes, presenting cysteine proteinases and/or cathepsin activities, may comprise important chemotherapy targets for different protozoal diseases [110]. TEM, particularly employing cytochemical [111] or immunocytochemical detection of markers, enzymes [112, 113] or their inhibitors such as cystatins [114] may be instrumental in the elucidation of their mode of action. The parasite endocytic/exocytic pathways may be affected in drug-treated parasites and TEM, using gold-labelled proteins, demonstrated that these pathways were down-regulated in dibucaine-treated *T. cruzi* [115].

The autophagic vacuole formation has been approached by TEM and cytochemistry [116, 117], but the phagophore assembly site was not ultrastructurally characterized yet [118]. Since many antiparasitic drugs trigger autophagic responses, the fine structural, cytochemical and analytical description of the forming compartments may help understanding the mode(s) of action of candidate or lead compounds [119].

ENDOPLASMIC RETICULUM AND GOLGI APPARATUS

EBIs were shown to produce prominent alteration of the endoplasmatic reticulum (ER) in trypanosomatid parasites [7, 8, 25], including the formation of myelin-like figures, which are often associated to oxidative stress and autophagy. Macroautophagy is mediated by ER cisternae enveloping portions of the cytoplasm [120, 121].

Cytosolic membrane arrangements resembling autophagic structures, leading to formation of autophagosomes, were also observed in *T. cruzi* treated with the combination of ketoconazole with the LPA edelfosine [69]. The formation of these cytosolic and mitochondrial membranar structures suggests that edelfosine induces parasite death through an autophagic process [8].

Drugs targeting the cytoskeleton can alter the ER, Golgi apparatus and vesicle distribution in most cell types. Leishmanial ER is found underneath the subpelicullar microtubules, associated to the parasite plasma membranes and eventually entering between them [122]. Vinblastine-selected *Leishmania* may present ER cisternae not only contacting the plasma membrane, but also protruding it outward (Fig. (**11**)).

Autophagic vacuoles may be mistaken for other functional ER configurations. In mammalian cells macroautophagy is mediated by ribosome-free ER cisternae. Therefore cisternae displaying bound ribosomes may erroneously be deduced to form autophagic vacuoles [123]. Nevertheless it must be kept in mind that parasite ER-controling enzymes under the effect of microbicidal compounds may present altered autophagic patterns and, possibly ribosome-preseting ER can give rise to *bona fide* autophagosomes (Figs. (**11**) and (**12**)). ER cisternae often give rise to myielin-like figures as in ketoconazole-treated *L. amazonensis* (Fig. (**11**)). Different compounds affect the Golgi apparatus organization and parasite vesicular traffic. *T. cruzi* epimastigotes treated with the β-lapachone derivative N3 present remarkable enlargement of Golgi cisternae (Fig. (**13**)).

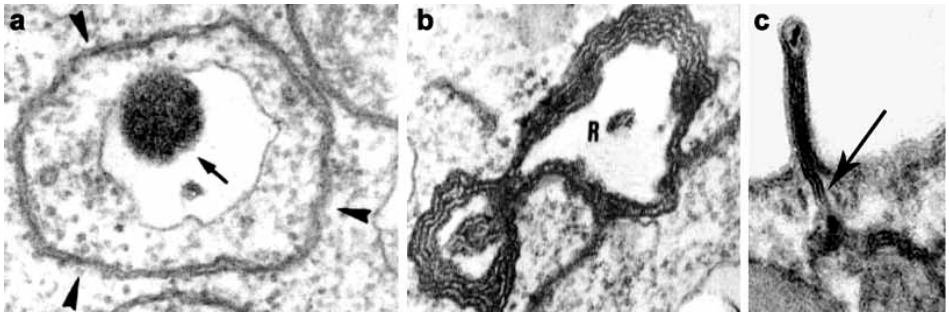

Fig. (11). Drug-induced ER alterations in *L. amazonensis.* Ketoconazole-treated (**a, b**) promastigotes presenting ER cistern (a, arrowheads) wrapping acidocalcisome (arrow). ER cisternae (R) forming myelin-like figure (**b**). Vinblastine-grown promastigote showing ER cistern (arrow) protruded outward pushing-out the parasite plasma membrane (**c**). Reproduced with permission.

ACIDOCALCISOME

Acidocalcisomes are acidic, calcium-storing compartments conserved in evolution which play important roles in parasite Ca^{2+} homeostasis intracellular pH regulation, polyphosphate metabolism and osmoregulation [124]. Acidocalcisomes comprise potential targets in antiparasitic chemotherapy as they display enzymes such as pyrophosphatase, not observed on mammalian organisms [125].

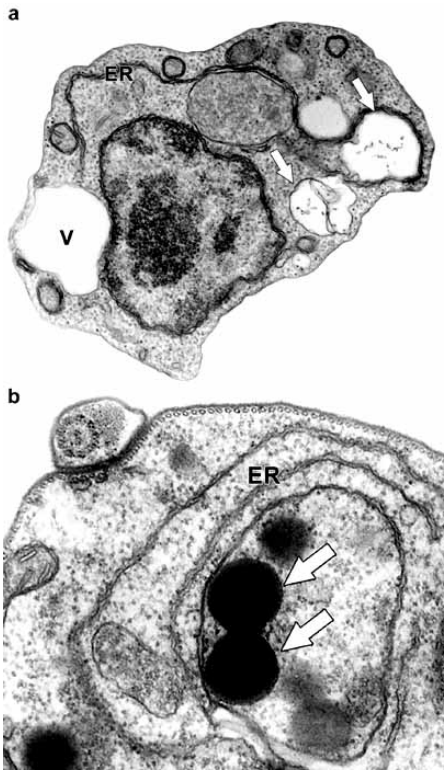

Fig. (12). Drug-induced macroautophagy in *T. cruzi*. Epimastigotes treated with the β-lapachone derivative N2 (**a**) and geranylgeraniol (**b**). Endoplasmic reticulum (ER) profiles surrounding cytoplasmic vacuoles (**a**, arrows). Extensive ER profiles lining cytoplasmic compartment presenting ribosomes and lipid droplets (arrows). Reproduced with permission.

Trypanosomatid and Apicomplexan parasites have large deposits of short-chain polyphosphates and their proliferation is inhibited by bisphosphonates, which are pyrophosphate analogues [63, 126-129]. Nitrogen-containing bisphosphonates such as alendronate, pamidronate and risedronate inhibit sterol biosynthesis at a pre-squalene level [130]. The selectivity of bisphosphonates towards the parasite as compared to mammalian cells may be due to their preferential accumulation in acidocalcisomes. Since risedronate inhibits sterol biosynthesis, the accumulation of lipid droplets in reservosomes could be a response of the parasite to the drug action, as a consequence of or accumulation of abnormal lipids and/or uptake of lipids from the culture medium. The presence of cytoplasmic lipid inclusions and autophagic vacuoles has also been noted in trypanosomatids treated with EBIs and is probably due to the accumulation of abnormal lipids and precursors in the cells [25, 64-68]. We have previously reported that the EBIs ketoconazole and terbinafine led to the formation of acidocalcisomes in *Leishmania* (Fig. (**14**)). The observation of intergradation between autophagosome compartments and acidocalciome-like compartments led us to infer that these organelles could be generated *via* endocytic/autophagic pathway [131]. These observations were faced with disbelief as the acidocalcisomes were re-

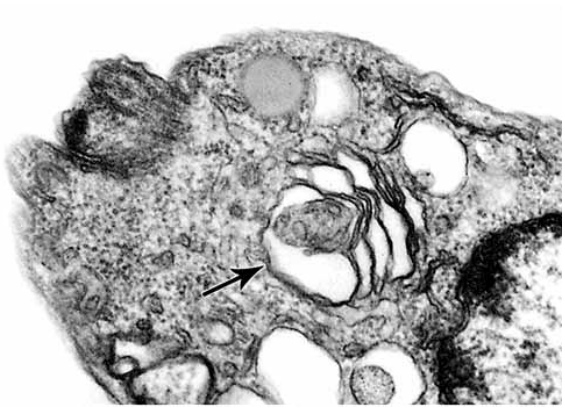

Fig. (13). Drug-induced alterations of the Golgi apparatus organization. *T. cruzi* epimastigote treated with the β-lapachone derivative N3 show enlargement of Golgi cisternae (arrow). Reproduced with permission.

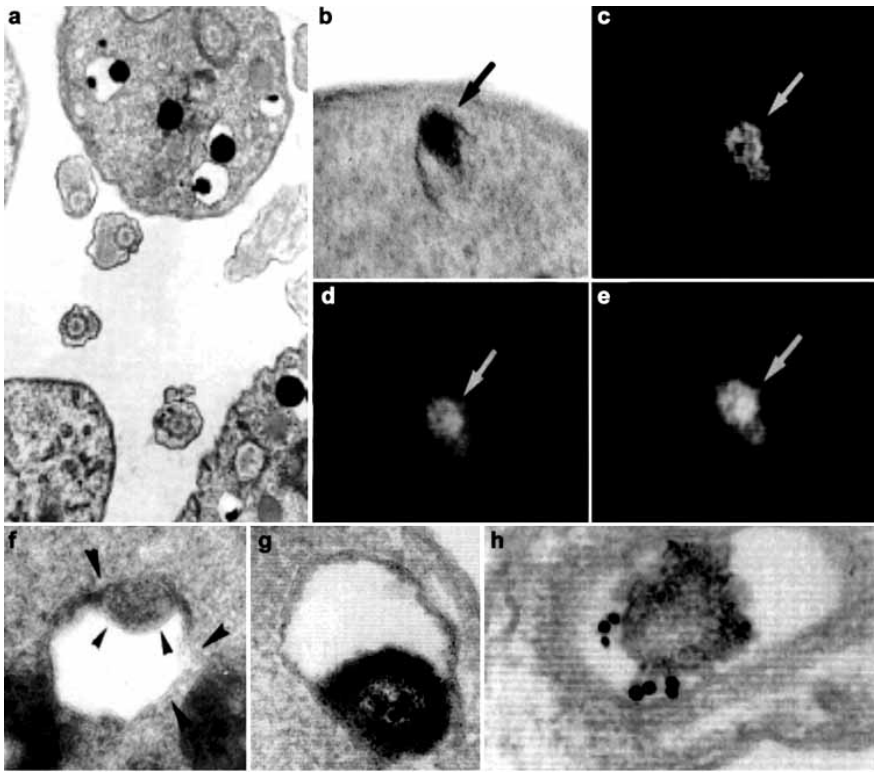

Fig. (14). Ultrastructural analysis of terbinafine-induced acidocalcisomes in *L. amazonensis.* Promastigotes, displaying an increased number and volume of acidocalcisomes (**a**). Electron spectroscopy imaging (**c-e**) of the acidocalcisome shown in (**b**). The elemental mapping revealed homogeneous phosphorus (**c**), oxygen (**d**) and calcium (**e**) distribution in the organelle core. TEM of acid phosphatase-positive acidocalcisome (**f**). Detection of horseradish peroxidase ingested by promastigote within acidocalcisome (**g**). Localization of gold-labeled cystatin C within acidocalcisome (**h**). Reproduced with permission.

ported not to take part in the *T. cruzi* transferrin endocytic pathway [132]. Although only gold-labeled tranferrin was tested at 1h incubation time in another parasite (*i.e. T. cruzi*), our data were not accepted at first. We used different markers and tracers at different time intervals and it indicated that the endocytic/autophagic pathway could give rise to acidocalcisomes. Later, the acidocalcisome formation from *Leishmania* multivesicular bodies was reported [133]. Interestingly, we also reported multivesicular body formation in the trans-Golgi network of EBI-treated *Leishmania* [25], and this organelle may be implicated in the autophagic (*vide infra*) process [134].

HYDROGENOSOME

Hydrogenosomes are compartments of endosymbiotic origin which pose interesting questions in Biology, comprising a unique model in parasite biochemistry as well as evolutionary and cell biology. This redox organelle of anaerobic microorganisms employs a pyruvate:ferredoxin oxidoreductase (PFOR) system for energy generation. Presumably because of prokaryote-like metabolic pathways, this organelle may be affected by many compounds, as demonstrated by electron microscopy [31]. Nevertheless drugs targeting this compartment may be of concern, since these organelles display a central role in the action of the drug of choice for trichomoniasis, the metronidazole [1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole]. This drug is reduced to its active form *via* either PFOR-mediated or a malate-dependent pathway [135]. As diminished hydrogenosomal function or ferredoxin gene transcription may lead to the failure in metronidazole activation, they are associated to metronidazole-resistant parasites [136, 137]. Such effects may be species-specific as DAB was shown to destroy hydrogenosomes in *Tritrichomonas foetus* [138], but not in *Trichomonas vaginalis* [139]. TEM study indicated the hydrogenosomal destruction, but during the organelle degradation the compartments were hardly identified (Fig. (**15**)). Thus post-embedding immunogold detection of the marker enzyme β-succinyl-coenzyme A synthetase was employed to unequivocally confirm the hydrogenosomal nature of the degraded compartments.

NUCLEUS

The ultrastructural analysis of the nuclear compartment may shed some light on the mechanisms underlying cell death. TEM revealed that type II topoisomerase inhibition leads to remarkable alterations of the nuclear compartment, including chromatin condensation and nuclear envelope distension [91]. Nuclear pyknosis and fragmentation are well established signs of PCD-I. TEM images of chromatin con-

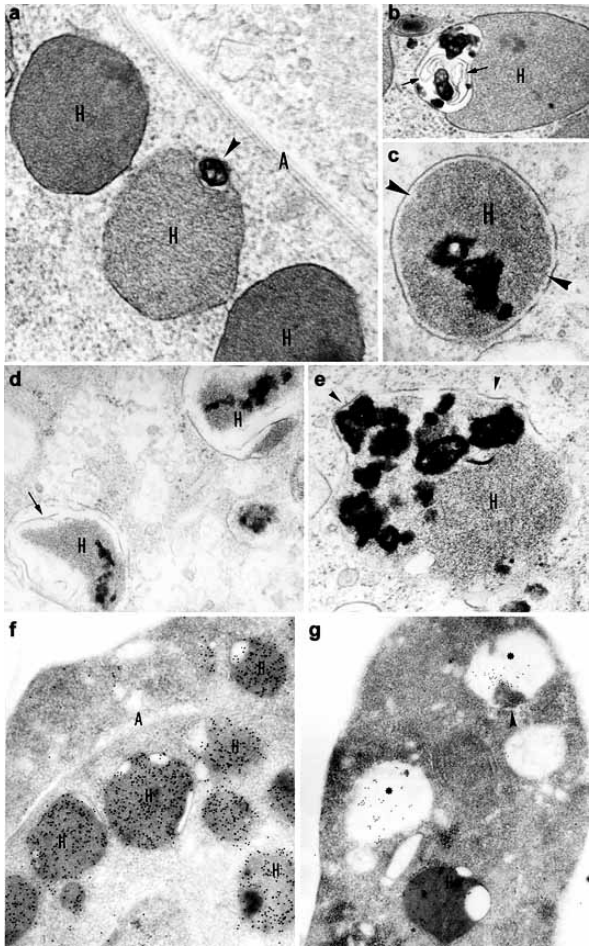

Fig. (15). Putrescine analogue effects upon trichomonad hydrogenosomes. Control *Tritrichomonas foetus* showing normal hydrogenosomes (H) in association with the axostyle component (A) of the cytoskeleton (**a**). The arrowhead indicates the organelle peripheral vesicle. DAB-treated *T. foetus* present free membranes within swollen peripheral vesicles (**b**, arrows), membrane detachment from organelle matrix (**c**, arrowheads) which displays electron dense deposits. At advanced stages numerous hydrogenosomes presented membrane damage (**d**, arrows) and matrix retraction, causing displacement from the organelle membranes. Hydrogenosome-like compartments (H) containing material rather similar to the hydrogenosomal matrix and were highly distorted, so hardly identified after DAB treatment (**d, e**). Immunogold detection of β-succinyl-coenzyme A synthetase in sections of control (**f**) and DAB-treated *T. foetus* (**g**). In control parasites, the gold labeling was mostly restricted to hydrogenosomes (H), whereas in DAB-treated parasites the gold labeling was also detected in vesicles (*). These compartments eventually presented immunoreactive, amorphous material with an electron density similar to that of the hydrogenosome matrix (arrowhead). Reproduced with permission.

densation must me interpreted with care, particularly as section planes cross the peripheral dense chromatin, apposed to the inner surface of the nuclear envelope. Unequivocal evidence of chromatin condensation must encompass considerable portion of nuclear compartment, preferentially in its central part (Fig. (**16**)). In these cases, fluorescence assays with DNA-staining with reagents such as DAPI may be useful, not only for the detection of DNA in whole cells, instead of a 50-70 nm-thick section, but also for the much higher number of cells observed in each sample. Therefore light microscopy data may corroborate the TEM findings.

The altered nuclear division pattern (karyokinesis) was demonstrated by TEM in suramin-treated *T. cruzi* [47]. TEM pattern of chromatin condensation was reported to discriminate caspase-dependent and -independent pyknosis [140]. Here again DNA laddering on agarose gels and phosphatidylserine flipping can corroborate PCD-I. Nevertheless it is noteworthy that caspase-dependent events may be involved in alternative mechanism of cell death such as necrosis or necroptosis [141].

The presence of over two nuclei (Fig. (**16**)) in protozoa such as trypanosomatid parasites strongly indicates impaired or down modulated cytokinesis, with unaltered karyokinesis [45].

TARGETING DISTINCT DEATH-STYLES

Parasitic protozoa display autophagic machinery, including an ATG8 homologue, and the processes may be implicated in starvation-induced differentiation of *T. cruzi* and therefore may furnish chemotherapy targets [142, 143]. As mentioned above, different antiparasitic compounds may trigger diverse death modes. Cell death mechanisms were extensively described *via* microscopy techniques and are still largely identified in microscopical approaches.

PCD is well characterized in higher eukaryotes and it was initially considered a synonym of apoptosis [28, 144]. Apoptosis (PCD-I) is a regulated process of self-induced cell death without inflammatory response, mainly characterized by cell shrinkage, DNA inter-nucleosomal fragmentation, phosphatidylserine exposure, plasma membrane blebing, apoptotic bodies formation, loss of mitochondrial membrane potential with cytochrome c and AIF release to the cytosol [145]. Autophagic cell death (PCD-II) involves the autophagosomal-lysosomal system with autophagosome formation, appearance of membranes surrounding organelles and cytosolic structures, without inflammatory response [146, 147]. Necrosis (PCD-III) involves mitochondrial damage, leading to ATP depletion, generation of ROS culminating in the rupture of plasma membrane [148]. Nevertheless, it must be pointed out that these cell death styles rather than individual entities are intricate crosstalking processes. The cells cultures with oligomycin and 2-deoxy-D-glucose can kinetically switch the suicidal program from apoptosis to necrosis [149]. In PCD-I, the organelle is swollen with inner membrane modeling and permeability transition pore (PTP) of the outer membrane allows the release of apoptosis-triggering molecules. In the second process (*i.e.* PCD-III) the PTP permeabilize the inner membrane causing remarkable enlargement of the organelle with consequent disrupture of the outer membrane [149]. Interestingly during the apoptosis-necrosis continuum, autophagic vacuoles are formed as an attempt to rescue the life of ATP-deprived cells. Cells undergoing oxidative stress by mitochondrial malfunctioning resulting in

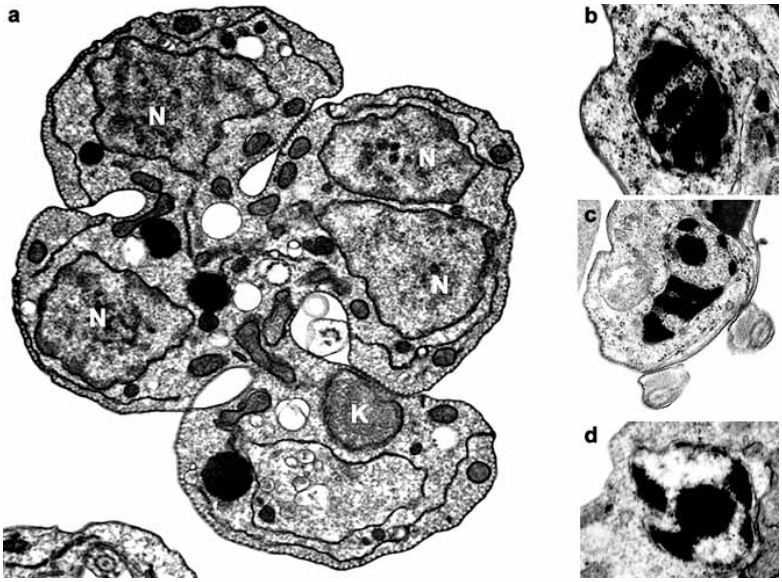

Fig. (16). Drug-induced nuclear alterations. Vinblastine-treated *L. amazonensis* promastigote (**a**) with lobulated appearance, due to truncated cytokinesis and multiple nuclei (N). Altered chromatin condensation pattern in *T. cruzi* trypomastigotes (**b, c**) and epimastigote (**d**) incubated with the β-lapachone derivative N2. Observe the pyknotic appearance of the chromatin (b-d, arrows). Reproduced with permission.

increased ROS production can eliminate the organelle by selective mitochondria autophagy (mitophagy) [150] and/or mitochondrial suicide (mitoptosis) [74, 148]. TEM may be used to identify apoptosis, necrosis, autophagy, as well as to discriminate the apoptotic mitoptosis of the mitochondrial inner and outer membranes [150].

PCD has been studied in *Leishmania* spp., as well as in amitochondrial protozoa such as *Entamoeba*, *Trichomonas* and *Giardia* [151]. A process called "apoptosis-like" has been demonstrated in protozoa, but its exact role in cell biology remains uncertain [152]. The interaction between different death pathways was suggested in protozoa such as *Blastocystis hominis*, *Tetrahymena termophila*, *Trichomonas* sp. and *Leishmania* spp. [7, 27, 153, 154]. However, the specific role of PCD in primitive eukaryotes is still controversial [152]. One of the hypotheses is that the behavior of the protozoa is altruistic, preventing uncontrolled proliferation and consequently increasing the success of the infection [155, 156]. Recently, PCD in the pathogenic protozoa *Cryptosporidium parvum*, *Leishmania* spp., *T. cruzi*, *Theileria* sp., *T. gondii* and *Plasmodium* sp. was associated with strategies of invasion, replication and evasion of the parasite from the host cell [157, 158]. In microorganisms, induction of PCD by different drugs has been described in bacteria, fungi and protozoa [7, 87, 159-163], and could be explored in the development of new drugs [156, 164].

CONCLUDING REMARKS

Taken together, the data briefly reviewed here unambiguously indicate the far-reaching conclusions regarding antimicrobials that may be obtained through electron microscopy studies on antiparasitic drugs, both on cellular drug targets and approaching their mechanism(s) of action. Nevertheless it does not suffice to employ modern, high performance equipment. Sharp-eyed, experienced researchers are required for avoidance of artifacts and misleading conclusions. This training demands countless long hours cautiously observing the effects of different compounds on distinct experimental models.

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ABBREVIATIONS

AIF	=	Apopotosis-inducing factor
DAB	=	1,4-diamino-2-butanone
DAPk	=	Death-associated protein kinase
EBIs	=	Ergosterol biosynthesis inhibitors
ER	=	Endoplasmatic reticulum
Leu-OMe	=	*L*-leucine methyl ester
LPAs	=	Lysophospholipid analogues
MDR	=	Multidrug resistance phenotype
MPT	=	Mitochondrial permeability transition
MVB	=	Multivesicular bodies
PCD	=	Programmed cell death
PFOR	=	Pyruvate:ferredoxin oxidoreductase

PTP = Permeability transition pore

ROS = Reactive oxygen species

SEM = Scanning electron microscopy

TEM = Transmission electron microscopy

TPA = 12-*O*-tetradecanoyl-phorbol-13-acetate

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