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Clomipramine kills *Trypanosoma brucei* by apoptosis

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ABSTRACT

Drug repositioning, *i.e.* use of existing medicals to treat a different illness, is especially rewarding for neglected tropical diseases (NTD), since in this field the pharmaceutical industry is rather reluctant to spend vast investments for drug development. NTDs afflict primarily poor populations in underdeveloped countries, which minimizes financial profit. Here we investigated the trypanocidal effect of clomipramine, a commercial antipsychotic drug, on *Trypanosoma brucei*. The data showed that this drug killed the parasite with an IC_{50} of about 5 μM. Analysis of the involved cell death mechanism revealed furthermore an initial autophagic stress response and finally the induction of apoptosis. The latter was substantiated by a set of respective markers such as phosphatidylserine exposition, DNA degradation, loss of the inner mitochondrial membrane potential and characteristic morphological changes. Clomipramine was described as a trypanothione inhibitor, but as judged from our results it also showed DNA binding capacities and induced substantial morphological changes. We thus consider it likely that the drug induces a multifold adverse interaction with the parasite's physiology and induces stress in a way that trypanosomes cannot cope with.

1. Introduction

Tropical Neglected Diseases (TNDs) are a diverse group of disorders with distinct clinical and etiological characteristics, broadly known by affecting mainly populations in low-income countries. Despite their important morbidity and mortality rates, historically the TNDs have not been subject of appropriate studies in the search and development of new drugs (Hotez et al., 2007). A complete revision carried out by Pedrique and collaborators (Pedrique et al., 2013) has shown that between 2000 and 2011, out of 850 new therapeutic products registered, only 25 were aimed for treatment or prevention of these TNDs. This inadequate support for research was pointed out by WHO as one of the majors obstacles in the control of neglected diseases (WHO, 2013a).

Among these TNDs, Human African Trypanosomiasis (HAT), also known as sleeping sickness, stands out as an important public health problem in Africa. As a vector-borne parasitic disease, it is caused by sub-species of *Trypanosoma brucei* and transmitted to humans by tsetse flies (*Glossina* spp.) (Brun and Blum, 2012). The three sub-species of the etiological agent are morphologically identical, but can be differentiated by their host specificity, epidemiology and genetic characteristics. There are three subspecies within the Brucei group: i. *T. brucei gambiense*, found in Western and Central Africa, is causing an anthroponotic disease and was during the last decade responsible for 98% of the total number of cases of HAT registered (WHO, 2013b); ii. *T.b. rhodesiense*, responsible for the minority of cases, has in contrast to *T.b. gambiense* a considerable animal reservoir that comprises various antelopes, carnivores and especially cattle. Transmission is usually between animals and flies and only occasionally to humans (Brun and Blum, 2012). iii. *T.b. brucei*, is not pathogenic for humans due to its susceptibility to lysis by a trypanolytic factor (TLF) within human serum, but able to infect domestic and wild animals; it is extensively used as a model in the study of HAT (Wheeler, 2010).

Although the number of infected individuals has significantly decreased in the last 20 years, around 7000 new cases have been

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recorded for 2014 on the African continent (Franco et al., 2014). Chemotherapy against *T. brucei* remains a problem. For over fifty years the treatment of HAT relied on suramin, pentamidine and the arsenical derivate melarsoprol. The only new drug registered in the past fifty years as an alternative for the treatment of second stage HAT was eflornithine. Although safer than the previous available treatments, monotherapy with eflornithine still presents serious problems such as adverse side effects, the difficulty of administration, the high price and the recent emergence of resistance (Burri, 2010). These problems have been reduced by a combination therapy of eflornithine/nifurtimox but it is still not ideal (Yun et al., 2010). Thus, new molecules and alternative therapies should be sought urgently for the treatment of HAT.

The discovery of new uses for old drugs, previously applied on the treatment of different diseases, is an interesting approach currently practiced by pharmaceutical industry in search of new medicines (Coura, 2009; Verma et al., 2005). Employing this approach, clomipramine, a tricyclic drug, originally used as an antipsychotic, has been tested and proved to be active against *Trypanosoma cruzi* (Rivarola et al., 2005; Strauss et al., 2013) and *T. brucei* (Richardson et al., 2009). Clomipramine and its analogs were identified as strong inhibitors of trypanothione reductase (Jones et al., 2010), which is an important enzyme on the redox metabolism of trypanosomes and a promising drug-target (Khan, 2007). However, detailed studies regarding the mechanism of death of clomipramine-treated parasites are still missing. Thus, the present study investigates the *in vitro* activity of clomipramine against *T. brucei,* as well as ultrastructural and biochemical alterations and the mechanism of cell death involved.

2. Methods

2.1. Chemicals

Clomipramine hydrochloride was purchased from Sigma-Aldrich (St. Louis, MI, USA). The fluorophores tetramethylrhodamine (TMRE), propidium iodide (PI) and dichlorofluorescein diacetate (DCFH) were obtained from Invitrogen (Karlsruhe, Germany) and the annexin V-Fluos kit from Roche (Mannheim, Germany). All other chemicals used were of the highest analytical reagent grade and obtained from Sigma Chemicals (Deisenhofen, Germany). Clomipramine was diluted in DMSO before each experiments, the final concentration of DMSO never exceeded 0.5% in all assays, which had no influence on the parasites in control experiments.

2.2. Parasites and cell culture

All of the experiments were performed with *T. brucei brucei* of the monomorphic strain EATRO 427 MITat 1.2 (VSG-variant 221). The bloodstream parasites were taken from frozen stabilates, cultivated in HMI-9 medium, and grown axenically at 37 °C in a humidified 5% CO_2 atmosphere, as described previously (Figarella et al., 2005). For each individual experiment, parasites at exponential growth phase (about 8×10^5 cells/mL) were counted and diluted to a cell density of 2×10^5 cells/mL in fresh HMI-9 medium.

2.3. Anti-proliferative activity

The determination of the anti-proliferative activity of clomipramine against *T. brucei* was performed by incubating the parasites (2×10^5 cells/mL) in HIM-9 medium in 24-well flat-bottom plates. The drug was added or not added at increasing concentrations. At different time-points, aliquots were aseptically taken and viable parasites were counted in a Neubauer hemocytometer. Parasites, which express normal motility and morphology, were considered as viable.

2.4. Cytotoxicity assay

In order to evaluate the cytotoxic effect of clomipramine against *T. brucei*, the phosphatase activity of treated and untreated parasites was measured as previously described (Bodley et al., 1995; Uzcátegui et al., 2007). Firstly, parasites were seeded at 2×10^5 cells/mL in 96-well flat-bottom plates and grown with or without clomipramine at concentrations between 1.4 and 14.2 μM. After 24 h of incubation at 37 °C, cell growth was stopped by addition of lysis buffer containing *p*-nitrophenylphosphate (20 mg/mL in 1 M sodium acetate, 1% Triton X-100, pH 5.5). The plates were incubated for 6 h at 37 °C and phosphatase activity was spectrophotometrically measured at 405 nm in a microplate reader (MRX II; Dynex Technologies, Middlesex, England). The concentration that diminished 50% of the absorbance value observed in the untreated control cells represented the IC_{50} (inhibitory concentration for 50% of the cells), and was determined by quadratic polynomial regression. Additionally, to assess the influence of an autophagy inhibitor on the activity of clomipramine against the parasite, we performed the same cytotoxicity experiment as described, but adding 0.5 μM of wortmannin to the medium of treated or untreated cells, respectively.

2.5. Mitochondrial-membrane potential

In order to evaluate the inner mitochondrial membrane potential, we conducted a TMRE staining. For this purpose, trypanosomes (2×10^5 cells/ml) previously treated for 24 h with 6 μM clomipramine were incubated in culture medium containing 25 nM of TMRE for 30 min at 37 °C, washed once in flow cytometry FC buffer (20 mM Na_2HPO_4, 5 mM NaH_2PO_4, 5 mM KCl, 1.3 mM MgSO4, 1 mM NaCl, 20 mM Glucose, pH 7.4) and immediately analyzed by flow cytometry on a BD FACSAria™ (Becton-Dickinson, Rutherford, NJ, USA) flow cytometer equipped with BD FACSDiva™ software. Valinomycin (100 nM) was used to cause depolarization of the inner mitochondrial membrane as a positive control. A total of 10,000 events were acquired each time in three independent experiments, in the region previously established as the one that corresponded to the parasites.

2.6. Reactive oxygen species (ROS)

To measure intracellular oxidative stress after clomipramine treatment, we used the fluorescent marker dichlorofluorescein diacetate (DCFH). For that, *T. brucei* (2×10^5 cells/mL) were incubated in the presence of 6 μM clomipramine for 24 h. After the incubation time, treated and untreated parasites were labeled with DCFH (10 μM) for 1 h at 37 °C. Afterwards, the fluorescence intensity of 10,000 cells was measured on a BD FACSAria™ flow cytometer equipped with BD FACSDiva™ software. The experiment was repeated at least three times independently.

2.7. Phosphatidylserine exposure

Phosphatidylserine exposure was detected using annexin-V FITC, a calcium-dependent phospholipid binding protein used as an apoptosis marker. Trypanosomes (2×10^5 cells/mL) were incubated in the presence or absence of 6 μM clomipramine for 24 h at 37 °C. Following the manufacturer's instructions, control and treated cells were washed in ligation buffer (HEPES 10 mM, pH 7.4, containing 140 mM NaCl and 5 mM $CaCl_2$) and incubated for 30 min at 4 °C with annexin V-FITC and counterstained with Propidium Iodide (1 μg/mL). Thereafter, 10,000 cells of each sample

were deployed on BD FACSAria™ flow cytometer equipped with BD FACSDiva™ software. The experiment was repeated at least three times independently.

2.8. Cell cycle analysis

In order to assess the interference of clomipramine on the cell cycle state of the parasite, we conducted a DNA content assessment by the propidium iodide (PI) staining method as previously described (Ferreira et al., 2011). Trypanosomes (2×10^5 cells/mL) incubated for 24 h at 37 °C in the presence or absence of 6 μM clomipramine were harvested, washed in buffer and incubated at 37 °C for 1 h in the same buffer containing digitonin (64 μM) and PI (10 μg/mL). Afterwards, a total of 10,000 cells were analyzed using a BD FACSAria™ flow cytometer equipped with BD FACSDiva™ software. The results obtained for treated cells were compared to histograms of untreated cells. According to the DNA content, expressed as relative fluorescence, nuclei were discriminated in different cell cycle phases (sub-G0, G0/G1, S/G2 and super-G2). Parasites classified as sub-G0 expressed nuclei with a reduced DNA content due to degradation processes. The experiment was repeated at least three times independently.

2.9. Cell volume determination

The cellular volume of treated and untreated parasites was evaluated as a function of Forward Scattering (FSC). For that purpose, trypanosomes (2×10^5 parasites/mL) treated or untreated for 24 h with 6 μM clomipramine were collected by centrifugation, washed in buffer, and directly analyzed on a BD FACSAria™ flow cytometer equipped with BD FACSDiva™ software. A total of 10,000 events were monitored in a region previously established for the parasites. The experiment was repeated three times independently.

2.10. DNA binding assay

In order to evaluate the capability of clomipramine to interact with DNA of trypanosomes, we adapted and performed a flow cytometry assay based on the properties of propidium iodide (PI). This probe is able to strongly intercalate into DNA and just then emits a fluorescent light (Banerjee et al., 2014). Trypanosomes (2×10^5 parasites/mL) were incubated in drug-free medium or in medium containing 6 μM clomipramine at 37 °C for 6 h. The parasites were then washed in FC buffer, before 1×10^5 parasites/mL were incubated at 37 °C for 1 h in the same buffer but containing digitonin (6 μM) and PI (10 μg/mL). Digitonin is able to permeabilize the plasma membrane of trypanosomes (Vercesi et al., 1991), allowing the entrance of PI through the lipid bilayer to interact with DNA. Following incubation, a total of 10,000 events were measured on a BD FACSAria™ flow cytometer equipped with BD FACSDiva™ software. The histograms of untreated cells were compared to those obtained for treated cells. A reduction of the fluorescence relative intensity was considered as lower intercalation of PI due to the previous intercalation of clomipramine to DNA. Alterations in the fluorescence for PI were quantified using an index of variation (IV) obtained by the equation IV = (MT − MC)/MC, where MT is the mean of fluorescence for treated parasites and MC the mean for control (*i.e.* untreated) parasites. A negative IV value corresponds thus to a lower intercalation of PI due to a previous intercalation of the drug of interest on the parasite's DNA.

2.11. Ultrastructural analysis

The effect of clomipramine on the ultrastructure of *T. brucei* was evaluated by transmission electron microscopy (TEM). Trypanosomes (2×10^5 parasites/mL) were treated with 6 μM clomipramine. After a 24 h incubation, parasites were harvested by centrifugation and washed 3 times in ice-cold PBS, followed by fixation in 2% (v/v) glutaraldehyde in 0.2 M cacodylate buffer containing 0.12 M sucrose (pH 7.4) for 1 h at 4 °C. Cells were washed 3 times with cacodylate buffer and incubated overnight in the same buffer. Afterwards, cells were first postfixed in 0.1 M cacodylate buffer containing 1.5% (w/v) osmium tetroxide for 1 h at 4 °C followed by a staining step in 0.1 M cacodylate buffer containing 0.5% (w/v) uranyl acetate for 1 h at RT. Samples were dehydrated in a graded series of ethanol (50% to 100%), propylene oxide and finally embedded in Agar 100 resin. Ultrathin sections were obtained using an ultramicrotome (OM U3, Reichert), stained with uranyl acetate and lead citrate, and monitored on a Zeiss EM 10 transmission electron microscope with TFP-camera using negative film Kodak Plus-X-Plan 120 (125 ASA, 60 × 70 mm). The parasites were analyzed and compared with untreated control cells.

2.12. Statistical analysis

All quantitative experiments were conducted in at least three independent experiments in duplicate. The statistical analyses were performed using the GraphPad Prism 5.0 software. The data were analyzed using one-way analysis of variance (ANOVA), and Dunnett's *post hoc* test was used to compare means when appropriate. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Clomipramine effects on parasite growth and viability

To analyze the cytotoxic effect of clomipramine on *T.b. brucei*, cells were treated with different concentrations of the drug, ranging from 0.03 μM to 28 μM under normal growth conditions (37 °C, 5% CO_2). Addition of different clomipramine concentrations caused a dose-dependent inhibition of cell growth, in which trypanosomes were killed by concentrations ranging from 7 μM to 28 μM. In addition, the IC_{50} value was determined by the method of Bodley et al. (Bodley et al., 1995), resulting in an IC_{50} value of 5.4 ± 0.23 μM (Fig. 1A). To investigate the role of autophagy during the clomipramine treatment, the experiment was performed under the same conditions but with addition of 0.5 μM wortmannin to the medium. This led to a slightly different IC_{50} value of 3.73 ± 0.43 μM, while in control parasites no cytotoxic effect was detectable in the absence or presence of 0.5 μM wortmannin (Fig. 1B). We thus consider autophagy not as a primary cause of cell death following clomipramine treatment.

3.2. Ultrastructural analysis

The ultrastructural changes induced by clomipramine treatment in bloodstream forms of *T.b. brucei* were verified by transmission electron microscopy. For specimen preparation, trypanosomes were incubated for 24 h in the presence or absence of clomipramine using regular cell culture conditions. Untreated control cells showed a normal ultrastructure containing a prominent nucleus and typical organelles structure and the plasma membrane including the VSG coat (Figs. 2–4). In contrast, parasites treated for 24 h with 6 μM clomipramine displayed a significantly altered ultrastructure. Most obvious was an increase of the rER and concomitantly the Golgi apparatus (Figs. 2 and 4), indicative for an increased protein biosynthesis. In addition, we found frequently dilated mitochondria containing virtually no cristae anymore, which are reduced in blood form trypanosomes anyway, but usually still present at a low level (Fig. 2). Another most prominent structure that frequently appeared was a usually huge membrane surrounded organelle containing plenty of cellular material of

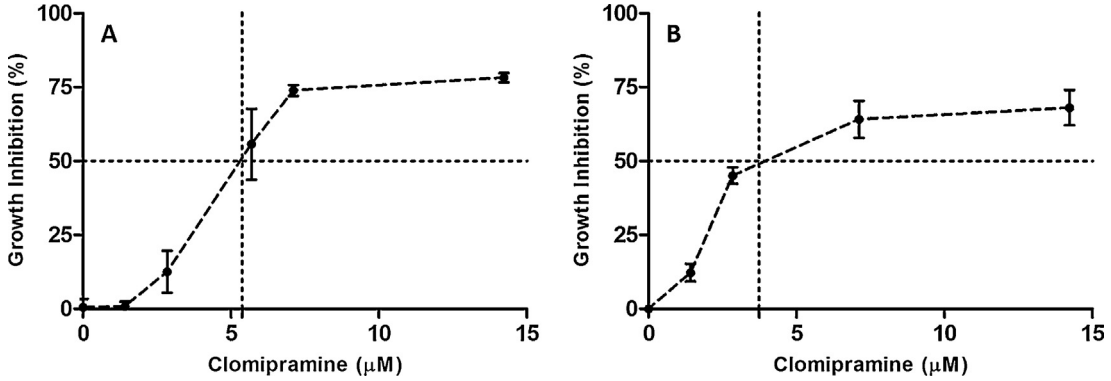

Fig. 1. Effects of clomipramine hydrochloride on the viability of cultured bloodstream forms of *Trypanosoma brucei*. Parasites were exposed to different concentrations of Clomipramine and the cytotoxicity was evaluated after 24 h as a function of phosphatase activity. The same experiment was conducted on the absence (A) and in the presence of the PI3 K inhibitor wortmannin 0.5 μM (B). Results are presented as percentage of growth inhibition compared to untreated parasites. The dotted lines represents approximately the IC_{50} values.

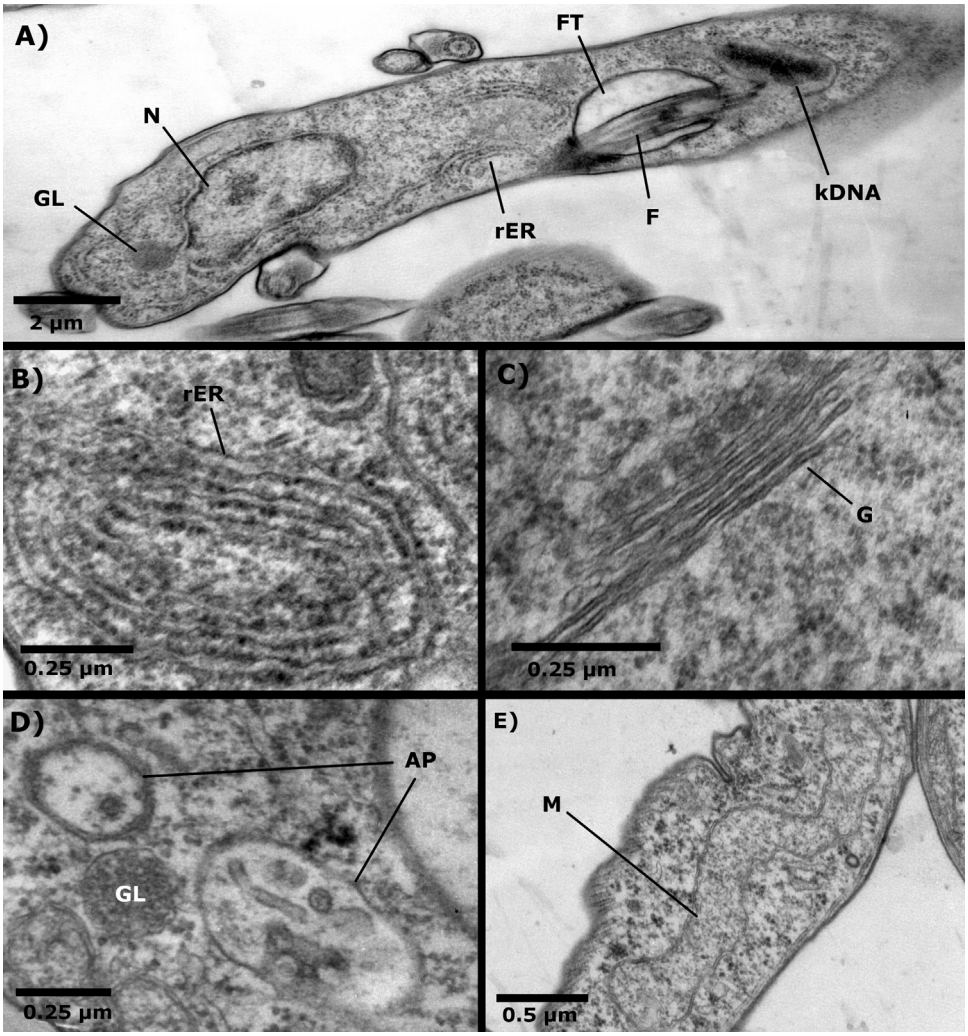

Fig. 2. Ultrastructural alterations (TEM) in bloodstream forms of *T. brucei* treated with clomipramine hydrochloride (6 μM). Parasites were treated for 24 h. (A) untreated parasites; (B–D) clomipramine-treated parasites showed (B) enlarged rough endoplasmatic reticulum (rER), (C) increased Golgi apparatus (G), (D) autophagosomes (AP) and (E) dilated mitochondrion (M). AP = autophagosome, F—flagellum, FT—flagellar pocket, G—Golgi apparatus, GL—glycosome, kDNA—kinetoplast DNA, M—mitochondrion, N—nucleus, rER—rough endoplasmatic reticulum.

organelle origin (Fig. 3). We also detected blebbing (Fig. 3), but as usual in trypanosomes, these blebs are fairly small, containing the VSG coat but never any cellular materials comparable to apoptotic bodies in higher eukaryotes. In fact this would be physically impossible because of the dense cytoskeleton formed by microtubules underneath the plasma membrane. We thus consider it likely that trypanosomes form apoptotic bodies inside the cell within these lysosomal-like organelle. Beside these differences,

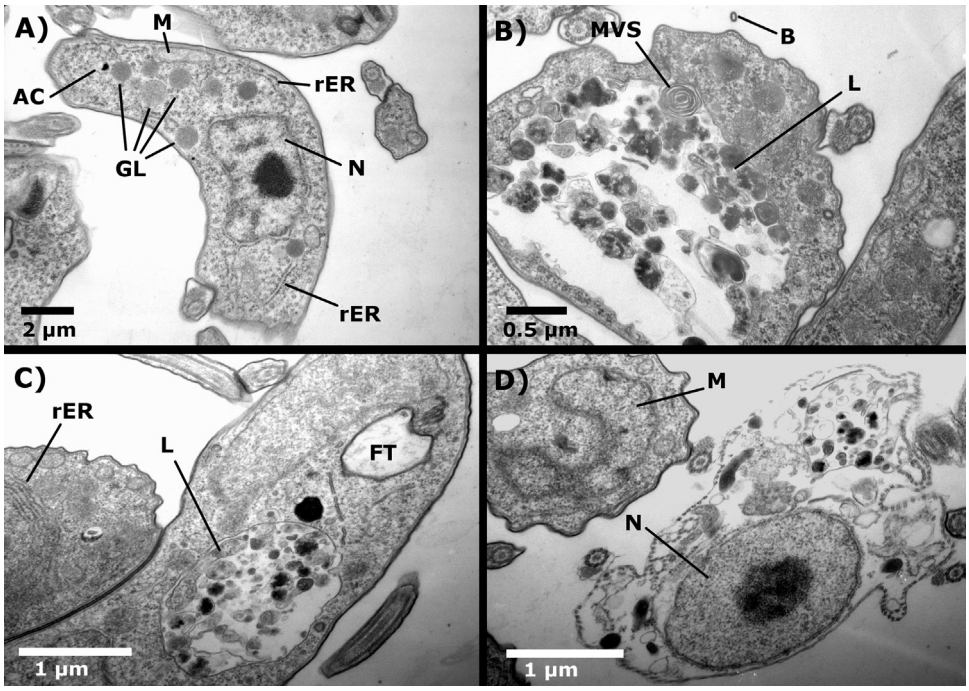

Fig. 3. Ultrastructural alterations (TEM) in bloodstream forms of *T. brucei* treated with clomipramine hydrochloride (6 µM). Parasites were treated for 24 h. (A) untreated parasites; (B–D) Clomipramine-treated parasites. B) and D) Lysosomes filled with plenty of cellular material of organelle origin, D) dead cell after clomipramine treatment. AC—acidocalcisome, B—blebbing, FT—flagellar pocket, GL—glycosome, L—lysosome, M—mitochondrion, MVS—multivesicular structure, N—nucleus, rER—rough endoplasmatic reticulum.

treated parasites looked rather normal in terms of cytosolic density, number and appearance of glycosomes as well as acidocalcisomes. We also detected autophagosomes, detectable by their typical double membranes. Although their number was not markedly higher than in control cells, they delivered their cargo into the huge lysosomal-like structures. Interestingly, we occasionally detected the content of the latter in a cell-free environment between trypanosomes (Fig. 3), so as if these remnants are released from dying cells. Since we never detected flagellar pockets filled with this material, we consider it likely that these remnants, like apoptotic bodies, are exposed after the final lysis of the cell (Fig. 3).

3.3. Cell volume determination

Regarding the cell volume of the parasite, trypanosomes treated as previously described were analyzed for Forward Scattering (FSC) by flow cytometry. The histograms in Fig. 5 show a significant reduction of FSC in the cell population treated with clomipramine. Comparing treated and untreated control cells, clomipramine led to a significant cell shrinkage, reducing the cell volume by 39%.

3.4. Mitochondrial-membrane potential

Since our results suggested that apoptosis may be involved in clomipramine-induced cell death, we also measured the mitochondrial-membrane potential ($\Delta\Psi m$). Our results show an intense decrease in TMRE fluorescence inside the mitochondrion after clomipramine treatment, which is directly related to the loss of $\Delta\Psi m$ (Fig. 6A). After 24 h of treatment with clomipramine, the parasites showed about 75% of positivity, *i.e.* a value very similar to the results obtained for valinomycin-treated cells (73%), which were used here as a positive control for mitochondrial depolarization.

3.5. Reactive oxygen species (ROS)

The previous results of mitochondrial depolarization prompted us to evaluate generation of reactive oxygen species (ROS) in *T. brucei* after clomipramine treatment, since oxidative stress is a possible result of mitochondrial dysfunction. Our findings indicate that clomipramine is able to induce ROS formation in the parasites, because treatment resulted in a percentage of positivity more than ten times higher than in control cells, ranging from 3.68% to 50.28% (Fig. 6B).

3.6. Phosphatidylserine exposure

To further check the mechanism of action of clomipramine on the parasite, we evaluated additional apoptotic hallmarks. Among several features of apoptosis, phosphatidylserine exposure at the outer leaflet of the plasma membrane is usually used as a distinctive characteristic. In the present work parasites were classified according to their staining by annexin-V and PI. Thus cells were characterized as early apoptotic cells (annexin-V positive, PI negative), late apoptotic cells (annexin-V positive, PI positive), and viable cells (annexin-V negative, PI negative). After treatment, most of the trypanosomes were found in late apoptotic stage (78%). About 10% of treated-parasites exhibited characteristics of early apoptosis, while viable cells comprise some 9% of the total population (Fig. 7). To distinguish between late apoptotic and necrotic cells, the morphology of treated trypanosomes were visualized by electron microscopy (Figs. 2 and 3).

3.7. Cell cycle analysis

Regarding the influence of clomipramine on the cell cycle and on DNA integrity of treated parasites, we permeabilized the cell membrane with detergent followed by PI staining. Our data suggest a strong increase on the number of cells at sub-G0 phase after treatment, which indicates loss of DNA integrity by the parasites (Fig. 8).

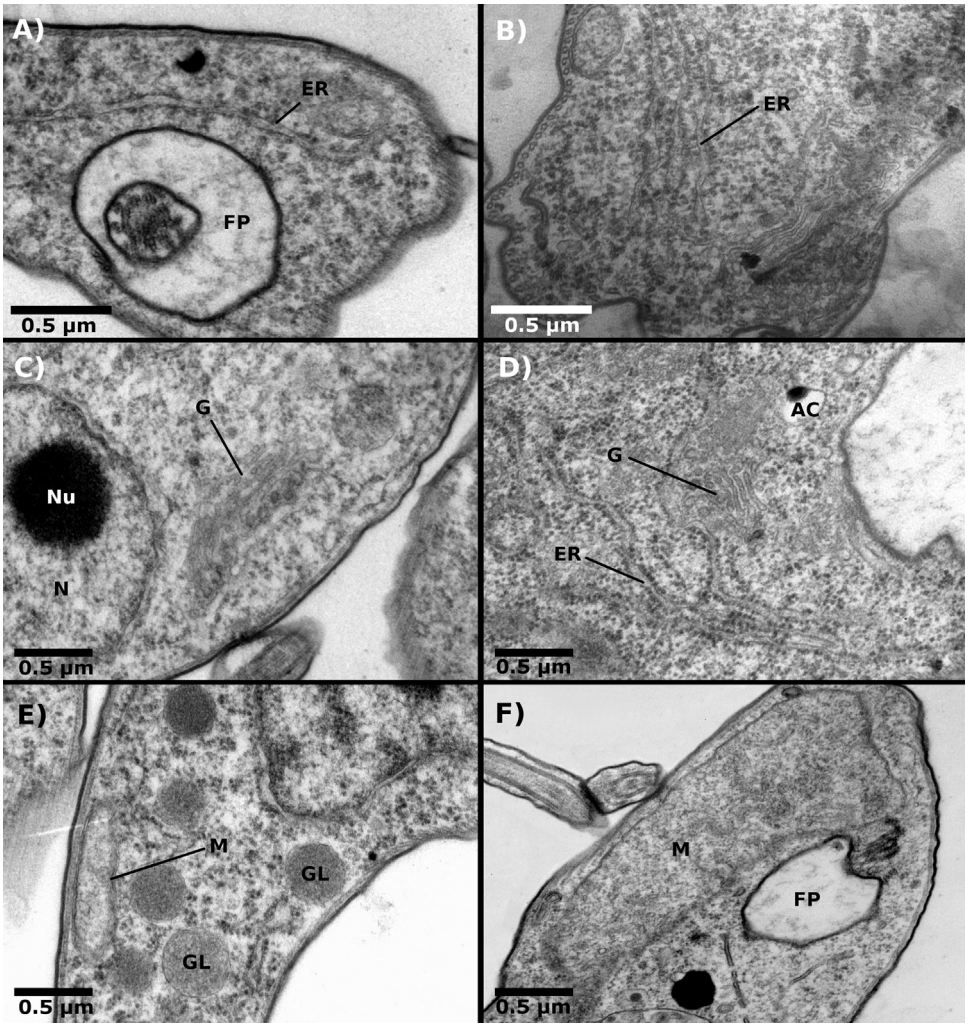

Fig. 4. Ultrastructural alterations (TEM) in bloodstream forms of *T. brucei* treated with clomipramine hydrochloride (6 μM). Parasites were treated for 24 h. (A, C, E)—untreated parasites; (B, D, F)—Clomipramine-treated parasites. (B, D) increase of rER membranes and Golgi stacks as compared to control cells (A, C). (F)—increase of the mitochondrion volume (dilatation) as compared to a control cell (E). AC—acidocalcisome, FT—flagellar pocket, G—Golgi apparatus, GL—glycosome, M—mitochondrion, N—nucleus, Nu—nucleolus, rER—rough endoplasmic reticulum.

Clomipramine induced DNA fragmentation in more than 40% of the population after 24 h of treatment. Additionally we observed a 50% reduction on the number of cells at G0/G1 phase, and a slight reduction at S/G2 phase after treatment. However, on the number of cells at the super-G2 level no change was observed, comparing treated and untreated parasites.

3.8. DNA binding assay

Our results regarding the involvement of DNA fragmentation on the mechanism of action of clomipramine prompted us to investigate the potential interaction of drug and DNA. Our results indicate that a 6 h pre-incubation of the parasite in medium containing clomipramine reduced the intercalation with PI and consequently the mean fluorescence in a statistically significant way. The index of variation (IV value, see Meth. Section 2.10) for mean fluorescence in pre-treated parasites changed to a value of −0.11 (or 11% respectively) thus showing a reduction of PI intercalation. In spite of that, when parasites were simultaneously incubated in the presence of clomipramine and PI no alteration in fluorescence could be observed and the index of variation (IV) were about zero in all replicates. The data suggest intercalation of clomipramine into DNA and thus occupation of binding sites for PI. However, PI seems to possess a higher affinity for DNA and will successfully compete if both compounds are given simultaneously.

4. Discussion

Despite multifold efforts to search for therapeutic alternatives to improve treatment, Human African Trypanosomiasis remains a serious health problem in various parts of sub-Saharan Africa. The currently available drugs are far from being ideal as they induce adverse and often severe side effects, are expensive, need hospitalization of patients, possess a complex treatment schedule and are increasingly ineffective because of growing rates of resistance (Burri, 2010).

In the past, discovery of new medicines has been done mainly by modifying the structure of effective molecules in a rational way to improve its efficacy. In general, this development is very expensive and time-consuming. In addition, the complete process of drug discovery, including pre-clinical and clinical studies, registration at drug administrations like FDA in the US, and introduction of new medicines to the market, may take up to 15 years and spend an average of 897.0 million US$ per drug (Verma et al., 2005). With regard to the specific problems of Neglected Tropical Diseases the issue is even more problematic, because the development of new

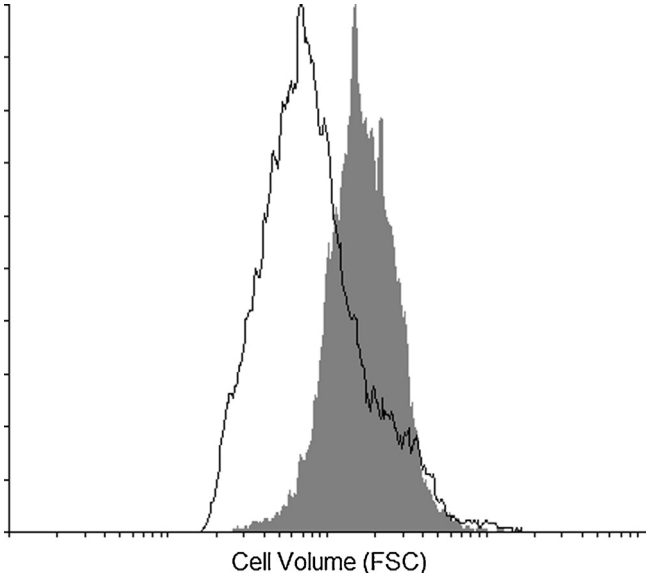

Fig. 5. Cell volume alteration of *T. brucei* treated with clomipramine hydrochloride for 24 h. The histograms assessed by flow cytometry show the relationship between the numbers of cells (counts) and Forward Scatter (FSC) considered as a function of cell volume. The gray-filled area represents untreated cells. Unfilled area represents parasites treated with Clomipramine (6 μM). Typical histograms of at least three independent experiments are shown.

drugs is unprofitable. Thus during the last decade only four new drugs were approved for treatment of NTDs, and none of them was against trypanosomiasis (Pedrique et al., 2013). Under such conditions, new approaches to find therapeutic alternatives for HAT and other NTDs are urgently needed.

The discovery of new applications for already existing and approved drugs, also known as drug repositioning, is a promising alternative for the currently used way of developing new drugs. For treatment of NTDs, several examples of this strategy are already in use: (1) allopurinol, originally conceived as an antineoplastic agent but further found effective on the treatment of Gout and Chagas' disease; (2) miltefosine, developed for the treatment of breast cancer but currently used against Leishmaniasis; and (3) amphotericin B, initially indicated against fungal infections but lately identified as a potent anti-protozoan drug. Based on that approach, we here report the promising inhibitory activity of clomipramine, a well-known antipsychotic drug that is often used against obsessive-compulsive disorder (OCD), against *T. brucei*.

In the present study, clomipramine exhibited strong effects *in vitro* against *T. brucei* bloodstream forms, inhibiting cell division and viability of the parasites. Data available in literature have already demonstrated that this tricyclic drug is lethal to trypomastigote and epimastigote forms of *T. cruzi* (De Barioglio et al., 1987), and a different strain of *T. brucei* (Richardson et al., 2009). Moreover, in previous studies clomipramine was also able to prevent formation of a chronic phase and effectively reduced mortality in *T. cruzi* infected mice (Rivarola et al., 2005; Strauss et al., 2013).

Our findings indicate a dose-dependent growth inhibition of *T. brucei* by clomipramine companied by an IC_{50} equal to 5.4 μM, a value virtually identical to the one previously obtained by Richardson et al. (IC_{50}: 5.04 μM; 13), though performed with a different strain, another methodology and an incubation time three times higher than ours. Clomipramine was also part of a recent analysis of promising drug candidates for sleeping sickness (Kaiser et al., 2015). Since all available data show its potency for treatment of HAT, we believe that the mode of action becomes an indispensable information for application.

To analyze the mechanism of action, we performed some experiments in order to identify the cell death pathway involved in the clomipramine depending killing of the parasites. According to Jones (Jones et al., 2010), the activity of clomipramine and other tricyclic compounds are generally associated with its action as a trypanothione reductase (TR) inhibitor, possessing an IC_{50} of 11.1 μM or 3.4 μM against the *T. cruzi* or the *T. brucei* TRs, respectively. This enzyme is involved in first line antioxidant defense on trypanosomatids, being particularly critical on the maintenance of the thiol redox balance. Since in most organisms the anti-oxidant defense relies mainly on glutathione instead of trypanothione in trypanosomatids, it is a promising target for drug-development against trypanosomiasis (Jones et al., 2010; Krauth-Siegel et al., 2008).

Our results evidenced strong mitochondrial depolarization and ROS generation after treatment, indicating an intense oxidative stress induced by clomipramine in *T. brucei*. These findings would be consistent with TR inhibition. In addition, a considerable dilatation of the mitochondrion of trypanosomes was observed on the ultrastructural level (Figs. 2 and 3). This substantial increase in volume, can be understood as a physiological response to the

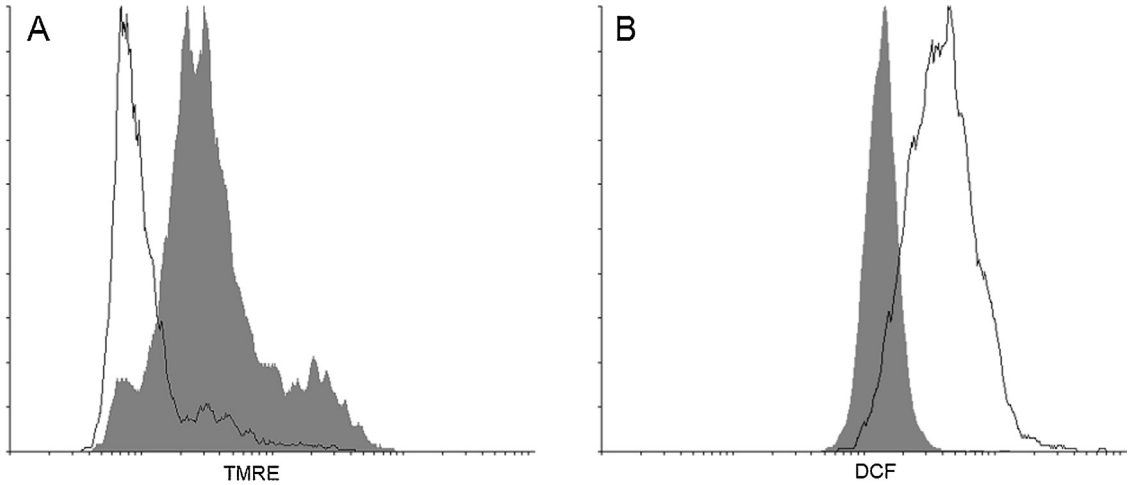

Fig. 6. Evaluation of mitochondrial-membrane potential ($\Delta\Psi$) and measurement of intracellular reactive oxygen species (ROS) level in *T. brucei* bloodstream forms after 24 h clomipramine hydrochloride treatment. (A) Parasites were stained with the fluorescent probe TMRE (2.5 nM) and analyzed by flow cytometry. (B) Parasites were stained with the probe DCFH (5 μM) and analyzed by flow cytometry. Dark gray-filled area represents untreated cells. Unfilled area represents parasites treated with clomipramine (6 μM). Typical histograms of at least three independent experiments are shown.

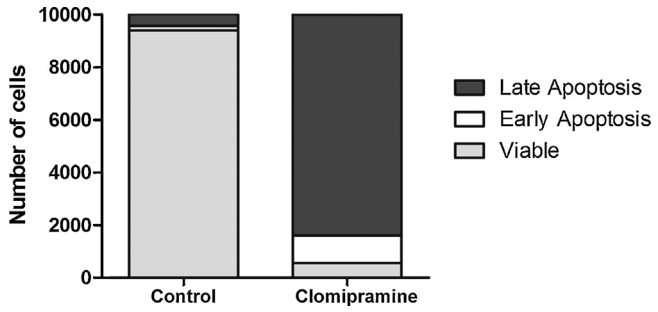

Fig. 7. Exposure of phosphatidylserine in *T. brucei* following a 24 h treatment with clomipramine hydrochloride. Parasites were treated with clomipramine (6 μM) for 24 h and analyzed by flow cytometry and classified regarding the cell death profile according to differential co-staining with annexin-FITC and PI (Viable cells: FITC−/PI−; Early apoptotic cells: FITC+/PI−; Late apoptotic cells: FITC+/PI+). Representative results of three independent experiments are shown.

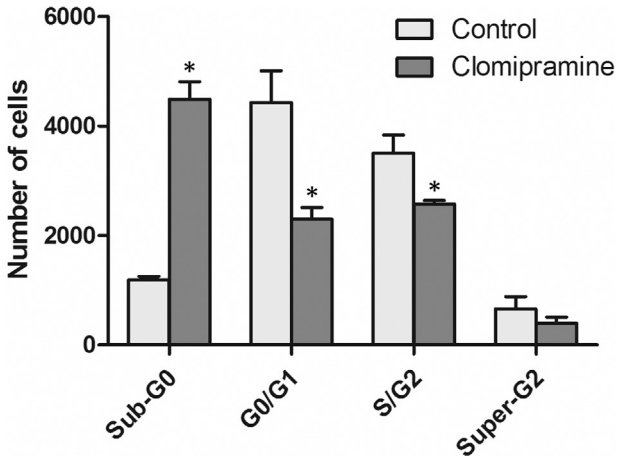

Fig. 8. Cell cycle analysis of *T. brucei* bloodstream forms treated with clomipramine hydrochloride (6 μM) for 24 h. Parasites after treatment were permeabilized, stained with PI (10 μg/mL) and analyzed by flow cytometry. The bars show the median ± standard deviation of number of parasites found in each cell cycle phase in three independent experiments. * $p \leq 0.05$.

mitochondrial membrane depolarization. It was suggested that de-energized mitochondria suffer from an imbalance of potassium movement, resulting in an influx of K+ and consequently a swelling mitochondrion (Safiulina et al., 2006). These mitochondrial volume alterations may lead to ROS formation (Juhaszova et al., 2004), consistent with our results.

A compromised redox homeostasis is one of the main causes for apoptosis in eukaryotes (Galluzzi et al., 2014). Besides this oxidative stress phenomenon, clomipramine induced also some other obvious alterations in *T. brucei*. Following treatment, parasites showed intense DNA fragmentation, cell shrinking and phosphatidylserine exposure. These features together with plasma membrane blebbing and formation of apoptotic bodies, are major hallmarks of apoptosis. The induction of DNA fragmentation and consequently initiation of apoptosis has been already verified previously in T-lymphocytes following clomipramine treatment (Xia et al., 1998). In trypanosomes blebbing is observed on the EM level, but because of the cytoskeleton organization blebs do not contain cellular structures. As shown in Fig. 2, we detected frequently organelle structures full of membrane surrounded remnants of cellular material. These degradation bodies are not released via the flagellar pocket, since we never observed these structures within the flagellar pocket, but are exposed to the surroundings by cell lysis. We are thus tempted to speculate that these organelle-like structures represent the apoptotic bodies of trypanosomes. As in the mammalian system, where apoptotic bodies are taken up by macrophages without inducing inflammation processes, this could also happen in natural infections as part of cell density regulation (Van Zandbergen et al., 2010).

Considering our previous results of DNA fragmentation after clomipramine treatment and knowing that compounds like clomipramine which possess a planar multi-cyclical structure, are likely to act as DNA intercalators (Snyder et al., 2004), we also tested whether or not clomipramine is able to interact with the DNA of *T. brucei*. Such interaction has been recently verified for other tricyclic antidepressants with DNA from mammalian cells (Yaseen et al., 2014) and we could confirm it here.

The maintenance of plasma membrane integrity observed on the majority of treated parasites, assessed by ultrastructure analysis (TEM), allowed us to exclude the possibility of cell death occurring by classical necrosis, which is well characterized by an increase in cell volume, plasma membrane rupture, and subsequent loss of intracellular content (Chaabane et al., 2013). Additionally, the ultrastructural analysis of treated parasites also revealed signs of autophagy, which was evidenced by an increase of autophagosomes (Menna-Barreto et al., 2009a,b). Together with the observed increase of rER membranes and Golgi stacks, we consider this cell behavior as a reaction to cope with the stress situation induced by clomipramine. However, since there is no sign of an autophagic cell death, *i.e.* a large increase of lysosomal structures, we interpret the described cellular responses as a cell survival strategy, which eventually fails because of the ongoing production of ROS and the compromised DNA functionality due to the intercalation process (Edinger and Thompson, 2004; Galluzzi et al., 2014; Krysko et al., 2008). This is also supported by the influence of wortmannin, a well know PI3 K inhibitor able to interfere on autophagosome formation (Yang et al., 2013), even so the effect is not very pronounced.

Altogether our findings indicate that clomipramine induces extensive oxidative stress by TR-inhibition and DNA binding. These effects lead to DNA-fragmentation, PS exposure, cell volume reduction, inhibition of the cell cycle, blebbing and formation of intracellular apoptotic bodies, which strongly suggest that the drug induces apoptosis. Autophagy seems to be initially triggered to rescue the cell, but end up contributing to cell demise. This mixed death profile has been already verified in trypanosomes treated with different chemotherapeutical agents (Lazarin-Bidóia et al., 2013; Menna-Barreto et al., 2009a,b), however the signaling pathway by which this cross-talking takes place on the parasite is still poorly understood.

5. Conclusion

In conclusion, our results corroborate the selective inhibitory activity of clomipramine against *T. brucei*, the causative agent of sleeping sickness. Regarding the mechanism of action, clomipramine seems to present a complex multi-target activity. Besides the already described inhibition of trypanothione reductase, our findings suggested that the drug can also directly interact with the parasite's DNA. Altogether, these effects led to ROS production, loss of the inner mitochondrial membrane potential, exposition of phosphatidylserine, and eventually induced apoptosis. Moreover our data indicate also the involvement of autophagic components, probably as a stress response. Taken together, the results herein achieved support further studies on the use of clomipramine, an already registered anti-depressant, as a promising chemotherapeutic agent against Human African Trypanosomiasis.

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Competing interests

All the authors declare no competing interests.

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