Universidade Estadual de Maringá
Centro de Ciências Biológicas
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Área de Concentração: Biologia Celular e Molecular

JEAN HENRIQUE DA SILVA RODRIGUES

Aspectos celulares e bioquímicos do mecanismo de ação do antidepressivo Clomipramina em protozoários tripanosomatídeos

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Março - 2017

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas (Área de concentração – Biologia Celular e Molecular) da Universidade Estadual de Maringá para a obtenção do grau de Doutor em Ciências Biológicas.

Orientador: Prof. Dr. Celso Vataru Nakamura

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FOLHA DE APROVAÇÃO

Jean Henrique da Silva Rodrigues

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Orientador: Prof. Dr. Celso Vataru Nakamura

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Aprovado em ___/___/___

BIOGRAFIA

Jean Henrique da Silva Rodrigues, nascido em Maringá em 16/01/1989, é filho de José Mendonça Rodrigues e Maura da Silva Rodrigues, sendo o mais novo de cinco filhos. Graduou-se em Ciências Biológicas na Universidade Estadual de Maringá em 2010, obtendo láurea acadêmica. Ao longo de sua graduação desenvolveu pesquisas envolvendo ecologia e microbiologia ambiental. Desde o início do mestrado em 2011, passou a integrar o Laboratório de Inovação Tecnológica no Desenvolvimento de Fármacos e Cosméticos, onde sob orientação do Prof. Dr. Celso Vataru Nakamura desenvolveu pesquisas na busca de substâncias naturais e sintéticas biologicamente ativas contra protozoários, bactérias, fungos e células tumorais. Em seu projeto de doutorado Jean Henrique se dedicou ao estudo do reposicionamento do antidepressivo Clomipramina contra *Leishmania amazonensis* e *Trypanosoma brucei,* agentes causadores da leishmaniose e da tripanossomíase africana respectivamente.

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E a todos aqueles não mencionados que também contribuíram com este trabalho.

Muito Obrigado!

" Sou biólogo

e viajo pela savana do meu país.

Nessas regiões encontro gente que não sabe ler livros.

Mas que sabe ler o mundo.

Nesse universo de outros saberes,

sou eu o analfabeto."

Mia Couto

Biólogo e Poeta Moçambicano

APRESENTAÇÃO

Em consonância com as regras do Programa de Pós-Graduação em Ciências Biológicas esta tese é composta por um resumo geral do trabalho limitado a 3 páginas em português e em inglês, e dois artigos de pesquisa científica original contemplando os resultados obtidos ao longo do desenvolvimento do doutorado. Ambas as publicações envolvem o reposicionamento do antidepressivo clomipramina como potencial agente contra duas Doenças Tropicais Negligenciadas. O primeiro artigo, desenvolvido em cooperação com o Prof. Michael Duszenko (UniTübingen/Alemanha) durante período de doutorado sanduíche, envolve a avaliação da atividade e do mecanismo de ação da clomipramina em *Trypanosoma brucei*, tendo sido publicado no *International Journal of Medical Microbiology* (ISSN: 1438-4221/ Impact Factor JCR: 3.89). O segundo manuscrito, apresenta o potencial reposicionamento do fármaco para o tratamento da leishmaniose, sendo descritas as atividades, alterações bioquímicas e ultraestruturais em *Leishmania amazonensis* tratadas por clomipramina. Sendo o segundo artigo redigido de acordo com as normas da revista *Biochemical Pharmacology* (ISSN*:* 0006-2952/ Impact Factor JCR: 5.091), e será submetido posteriormente.

RESUMO GERAL

INTRODUÇÃO

As Doenças Tropicais Negligenciadas (DTN) são um grupo de severas condições debilitantes, caracterizadas pelo amplo impacto socioeconômico e por afetarem principalmente populações mais pobres. Apesar dos altos índices de mortalidade e morbidade das DTN faltam estudos apropriados na busca de novos medicamentos, possivelmente devido às baixas expectativas de lucros.

Dentre as DTN, as parasitoses causadas por protozoários da ordem Kinetoplastida são reconhecidas por afetarem milhões de pessoas e animais em todo o mundo. Os mais importantes agentes etiológicos neste táxon incluem protozoários dos gêneros *Leishmania*, responsáveis por diversas manifestações clínicas denominadas leishmanioses, e *Trypanosoma*, que incluem os agentes etiológicos das tripanosomíases humanas. Sendo na África subsaariana *T. brucei* causador da doença do sono, enquanto na América latina *T. cruzi* é responsável pela doença de Chagas. A quimioterapia atualmente disponível para estas doenças permanece um problema, uma vez que não existem vacinas e que os poucos medicamentos disponíveis tem eficácia restrita.

Na busca de novos tratamentos para DTN o reposicionamento de fármacos surge como uma alternativa promissora. Esta abordagem mais barata e rápida consiste na identificação de novos usos para fármacos originalmente registradas para outros fins, tendo sido aplicada com sucesso no registro dos fármacos Miltefosina e Anfotericina B para o tratamento da leishmaniose, substâncias estas inicialmente utilizadas como antitumoral e antifúngico, respectivamente.

Baseando-se nesta estratégia, a Clomipramina, um fármaco atualmente empregado na terapêutica do Transtorno Obsessivo Compulsivo, foi identificada como um potente inibidor da Tripanotiona redutase (TR) com promissor uso no tratamento da doença de Chagas. Considerando que a TR é uma enzima primordial e exclusiva do metabolismo antioxidante de tripanosomatídeos, o presente trabalho visa avaliar o reposicionamento do antidepressivo Clomipramina (clomi) para o tratamento da leishmaniose e da doença do sono, bem como descrever as alterações ultraestruturais a elucidar o mecanismo de ação do fármaco nos parasitos.

MÉTODOS

O potencial de clomi foi avaliado *in vitro* contra duas espécies de protozoários flagelados: *Trypanosoma brucei brucei* (cepa EATRO 427 MITat 1.2), mantida em meio HMI-9 a 37 °C e 5% CO_2, e *Leishmania amazonensis* (cepa WHOM/BR/75/JOSEFA), cultivada em meio Warren a 25 °C. A atividade antiproliferativa de clomi foi determinada contra formas sanguíneas de *T. brucei*, por contagem direta e atividade de fosfatase como indicador de viabilidade. O efeito inibitório contra formas promastigotas de *L. amazonensis* foi estimada pelo ensaio de redução de XTT. O potencial contra formas amastigotas intracelulares foi aferido em macrófagos J774A.1 infectados com *L. amazonensis.* A citotoxicidade de clomipramina foi determinada em macrófagos J774A.1 usando o sal de tetrazólio MTT.

O mecanismo de ação do antidepressivo foi determinado em parasitas extracelulares incubados com o IC_{50} de clomi por pelo menos 24 h. Com o intuito de analisar o efeito em formas intracelulares de *L. amazonensis*, macrófagos previamente infectados também foram tratados por 24 h com clomi (IC_{50} e IC_{90}), células foram rompidas e os protozoários isolados por centrifugação diferencial. Alterações morfológicas e ultraestruturais em protozoários tratados foram observadas por microscopia eletrônica de varredura e transmissão. Parâmetros celulares relacionados ao metabolismo energético, integridade das membranas, ciclo celular, além da ocorrência de apoptose e autofagia, também foram analisados nestes parasitos, utilizando diversas técnicas, como citometria de fluxo, espectrofotometria e fluorimetria.

RESULTADOS E DISCUSSÃO

Nossos resultados evidenciaram o potencial inibitório de clomi contra os agentes etiológicos da leishmaniose e da doença do sono, com concentrações inibitórias para 50% do crescimento celular (IC_{50}) igual à 5,4 ± 0,23 µM (24 h), 8,31 ± 3,29 µM (72 h) e 15,45 ± 4,92 µM (48 h), respectivamente para *T. brucei*, promastigotas e amastigotas de *L. amazonensis*. Além de seu amplo uso clínico, a segurança e seletividade de clomi foi corroborada pela baixa toxicidade contra macrófagos (CC_{50}: 181,22 ± 8,04 µM).

Em termos de alvos celulares da ação de clomi, nossas descobertas indicam claro envolvimento mitocondrial. A despolarização da membrana mitocondrial surge como um efeito primordial no complexo mecanismo de ação do fármaco em ambos protozoários. Este comprometimento mitocondrial juntamente com a inibição da TR, enzima envolvida na redução do antioxidante tripanotiona, leva à intenso estresse oxidativo nos parasitos.

Adicional interação direta entre clomipramina e o DNA do parasito também foi detectada em *T. brucei.*

Dentre diversas consequências, o estresse oxidativo em promastigotas foi evidenciado como intensa lipoperoxidação, inclusões lipídicas e danos na membrana plasmática, efeitos estes ao menos parcialmente inibidos pelo uso do antioxidante N-acetilcisteína. Em amastigotas contudo, nem lipoperoxidação tampouco alterações na membrana foram observadas, possivelmente devido à particular resistência ao stress característica deste estágio do parasito. Outra importante diferença metabólica foi denotada ao analisar os níveis de ATP celular, que foram reduzidos em amastigotas mas se mantiveram constantes em promastigotas, apesar da despolarização mitocondrial induzida.

Na busca em restabelecer a homeostase vias de recuperação são ativadas na célula, sendo intensa autofagia observada em ambos protozoários. Interessantemente, quando o inibidor de autofagia wortmannin foi usado juntamente com clomi observou-se sensível aumento da atividade inibitória, indicando o papel recuperador desta via. Também como resposta adaptativa detectou-se também exacerbação da via exocítica em *L. amazonensis* tratadas com clomi.

Ao falhar na recuperação da homeostase celular, tais respostas findam por contribuir com a morte dos parasitos. Efeitos tardios observados incluem sinais típicos de morte celular programada (ex: fragmentação do DNA, exposição da fosfatidilserina, encolhimento do tamanho e atividade semelhante à caspase), sugerindo morte do protozoário por uma via semelhante à apoptose.

CONCLUSÃO

Tomados em conjunto, nossos resultados subsidiam estudos avançados de reposicionamento da clomipramina no tratamento da leishmaniose e doença do sono. O antidepressivo age seletivamente em *L. amazonensis* e *T. brucei* por uma via majoritariamente mitocondrial, induzindo intenso estresse oxidativo e, apesar das respostas adaptativas de autofagia e exocitose, finda por induzir nos protozoários morte celular programada semelhante a apoptose.

GENERAL ABSTRACT

INTRODUCTION

Tropical Neglected Diseases (TNDs) are a group of severe disabling conditions characterized by their social and economic impacts, and by affecting mainly people in the poorest 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, possibly due to the restrict profit expected.

Among the TNDs the parasitic diseases caused by protozoa belonging to order Kinetoplastida are known by affecting millions of people and animals all over the world. The most important etiological agents in this group are protozoa from genera *Leishmania,* which cause a broad range of diseases in the tropics and subtropics called leishmaniasis, and *Trypanosoma,* that includes causative agents of human trypanosomiasis. In sub-Saharan Africa, *Trypanosoma brucei* is responsible for causing sleeping sickness, and in Latin America, *Trypanosoma cruzi* causes Chagas' disease. Treatment for these diseases remains a problem, since vaccines are not available and chemotherapy is restricted to only a few drugs with limited efficacy.

On the pursuit of novel therapeutic alternatives for NTD, drug repurposing arises as a faster and cheaper approach. Also known as drug repositioning, it consists on the identification of new uses for medicaments originally approved for a different purpose. Based on that strategy, miltefosine and amphotericin B, drugs respectively conceived and anti-cancer and anti-fungus agents where later repurposed for the treatment of leishmaniasis.

Clomipramine, a drug initially conceived as an anti-depressant and currently used in the treatment of Obsessive Compulsive Disorder was found to be an efficient inhibitor of trypanothione reductase (TR) with promising use on Chagas disease. Considering that TR is an exclusive and essential enzyme on the antioxidant metabolism of trypanosomes, the present work evaluates the repurposing of the antidepressant clomipramine (clomi) for the treatment of leishmaniasis and Human African Trypanosomiasis (HAT), as well the ultrastructural and biochemical alterations involved on the mechanism of action of the drug on the parasites.

METHODS

The potential of clomi was evaluated *in vitro* against two species of flagellate protozoa: *Trypanosoma brucei brucei* (EATRO 427 MITat 1.2 strain), maintained in HMI-9 medium at 37 °C and 5% CO_2, and *Leishmania amazonensis* (WHOM/BR/75/JOSEFA strain), cultivated in Warren medium at 25 °C. The antiproliferative activity of clomi was assessed against bloodstream forms of *T. brucei*, using both direct counting and phosphatase activity as a viability sensor. The inhibitory activity against *L. amazonensis* promastigotes was assessed by the tetrazolium salt XTT. The activity against intracellular amastigotes was evaluated in J774A.1 macrophages infected with *L. amazonensis* promastigotes. The safety of clomi against mammal cells was determined in J774A.1 macrophages by MTT reduction assay.

The mechanism of action of clomi was determined in extracellular parasites incubated with the IC_{50} for at least 24 h. In order to analyze the effect in *L. amazonensis* intracellular amastigotes, infected macrophages were also treated for 24 h with IC_{50} and IC_{90}, afterwards parasites were isolated through host cell disruption and differential centrifugation. The ultrastructural and morphological alterations in clomi-treated parasites were observed by transmission and scanning electron microscopy. Cell parameters related to energetic metabolism, membrane integrity, cell cycle, plus the occurrence of apoptosis and autophagy were also analyzed in clomi-treated parasites, by using sort of different techniques, including flow cytometry, spectrophotometry and fluorimetry.

RESULTS AND DISCUSSION

Our results showed the inhibitory potency of clomi against the causative agents of leishmaniasis and HAT, presenting inhibitory concentrations for 50% (IC_{50}) of cell growth/viability equal to 5.4 ± 0.23 µM (at 24 h), 8.31 ± 3.29 µM (at 72 h) and 15.45 ± 4.92 µM (at 48 h), respectively for *T. brucei* bloodstream forms, promastigotes and amastigotes of *L. amazonensis*. Besides its wide use in the clinics, the safety of the drug was also assured by low cytotoxicity against host macrophages (CC_{50}: 181.22 ± 8.04 µM), what indicates the selective activity of clomi against the parasites.

In terms of cellular targets of clomi action, our findings clearly indicates deep mitochondrial involvement. The mitochondrial membrane potential depolarization seems to be a primordial step of the mechanism of action in both parasites. Together with the inhibition of TR, an enzyme involved on the recycling of the antioxidant trypanothione,

the impairment of mitochondrial potential led to ROS accumulation and end up in intense oxidative stress. Additional direct interaction between clomipramine and the parasite DNA has also been detect in *T. brucei*.

Among several consequences, the oxidative stress in promastigotes was evidenced as intense lipoperoxidation, accumulation of lipid droplets and disruption of cell membrane, all effects at least partially inhibited when cells were pre-treated with the antioxidant n-acetylcysteine. Interestingly, neither lipoperoxidation nor cell membrane disruption were observed in intracellular amastigotes, possibly due to the stress metabolic resistance innate of this parasitic stage. Another important metabolic difference was observed by analyzing the ATP levels, which were reduced in amastigotes but kept constant in promastigotes, although the mitochondrial depolarization induced by clomi.

In attempt to get homeostasis back cell makes use of recovery pathways, with remarked autophagy observed in both parasites. Interestingly, in *T. brucei* when wortmannin (autophagy inhibitor) has been simultaneously applied with clomi an increase in the inhibitory activity has been observed, possibly indicating its saving role. As an additional adaptive response the exacerbation of exocytosis was also detected in clomi-treated *L. amazonensis*.

The unsuccessfully effort to recover cell homeostasis terminates contributing to cell demise. Later effects observed in treated parasites includes typical features of programmed cell death (i.e. DNA fragmentation, phosphatidylserine exposition, cell shrinking and caspase-like activity) suggesting death by an apoptosis-like pathway.

CONCLUSION

Taken together our results supports further studies on the repurposing of Clomipramine on the treatment of leishmaniasis and HAT. The antidepressant selectively acts on *L. amazonensis* and *T. brucei* by a mitochondrial pathway mostly, inducing severe oxidative stress and, despite the adaptive responses of autophagy and activated exocytosis, parasites end up dying through an apoptosis-like programmed cell death.

FULL PAPER 1

RODRIGUES, JEAN HENRIQUE DA SILVA; STEIN, JASMIN; STRAUSS, MARIANA; RIVAROLA, HECTOR WALTER; UEDA-NAKAMURA, TÂNIA, NAKAMURA, CELSO VATARU; DUSZENKO, MICHAEL. Clomipramine kills Trypanosoma brucei by apoptosis.

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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 under-developed 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.

KEYWORDS: *Clomipramine; Apoptosis; Sleeping Sickness; Programmed Cell Death*

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. *Trypanosoma 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 7,000 new cases have been 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 *Trypanosoma brucei*, as well as ultrastructural and biochemical alterations and the mechanism of cell death involved.

METHODS

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.

Parasites and Cell Culture

All of the experiments were performed with *Trypanosoma 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 x 10^5 cells/mL) were counted and diluted to a cell density of 2 x 10^5 cells/mL in fresh HMI-9 medium.

Anti-proliferative Activity

The determination of the anti-proliferative activity of clomipramine against *T. brucei* was performed by incubating the parasites (2 x 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.

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 x 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 24h 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.

Mitochondrial-membrane potential

In order to evaluate the inner mitochondrial membrane potential, we conducted a TMRE staining. For this purpose, trypanosomes (2 x 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.

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 x 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.

Phosphatidylserine exposure

Phosphatidylserine exposure was detected using annexin-V FITC, a calcium-dependent phospholipid binding protein used as an apoptosis marker. Trypanosomes (2 x 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.

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 x 10^5 cells/mL) incubated for 24 h at 37°C in the presence of 6 µM clomipramine were harvested, washed in buffer and incubated at 37°C for 1h 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.

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 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.

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 this mean for control parasites (untreated parasites). A negative IV value corresponds to a lower intercalation of PI, probably due to a previous intercalation of the drug of interest on the parasite DNA.

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 x 70 mm). The parasites were analyzed and compared with untreated control cells.

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.

RESULTS

Clomipramine effects on parasite growth and viability

To analyze the inhibitory 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 culture conditions (37°C, 5% CO_2). Addition of different clomipramine concentrations caused a dose-dependent inhibition of cell growth, in which trypanosomes were completely abolished by concentrations ranging from 7 µM to 28 µM. In order to precise the range of inhibition on the metabolic viability of the parasites, 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 ± 0.23µM (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.

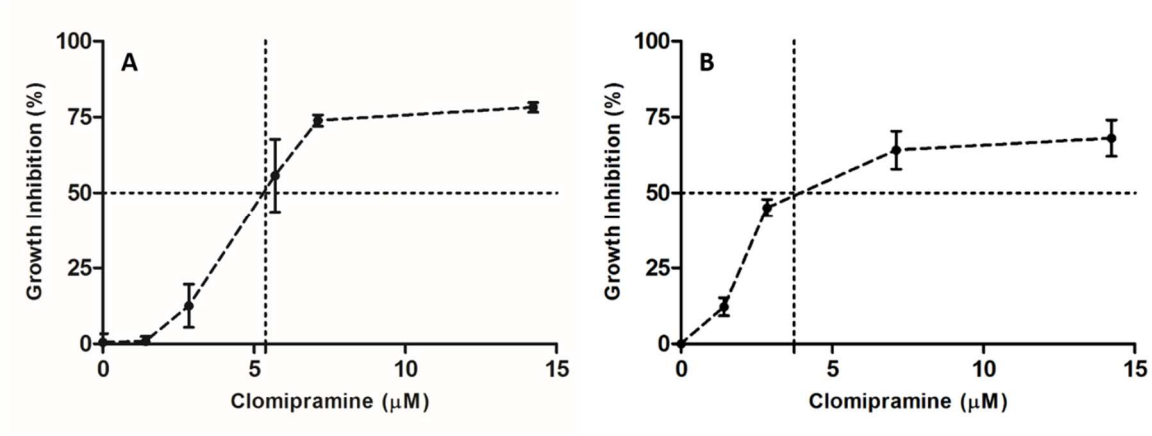


Figure 1. Effects of clomipramine hydrochloride on the viability of cultured bloodstream forms of *Trypanosoma brucei*. Parasites were exposed to different concentrations of Clomipramine and the cytotoxicity was evaluated after 24 h as a function of phosphatase activity. The same experiment was conducted on the absence (A) and on the presence of the PI3K 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.

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 (Fig. 2, 3 and 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 (Fig. 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 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 of 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, 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 either 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).

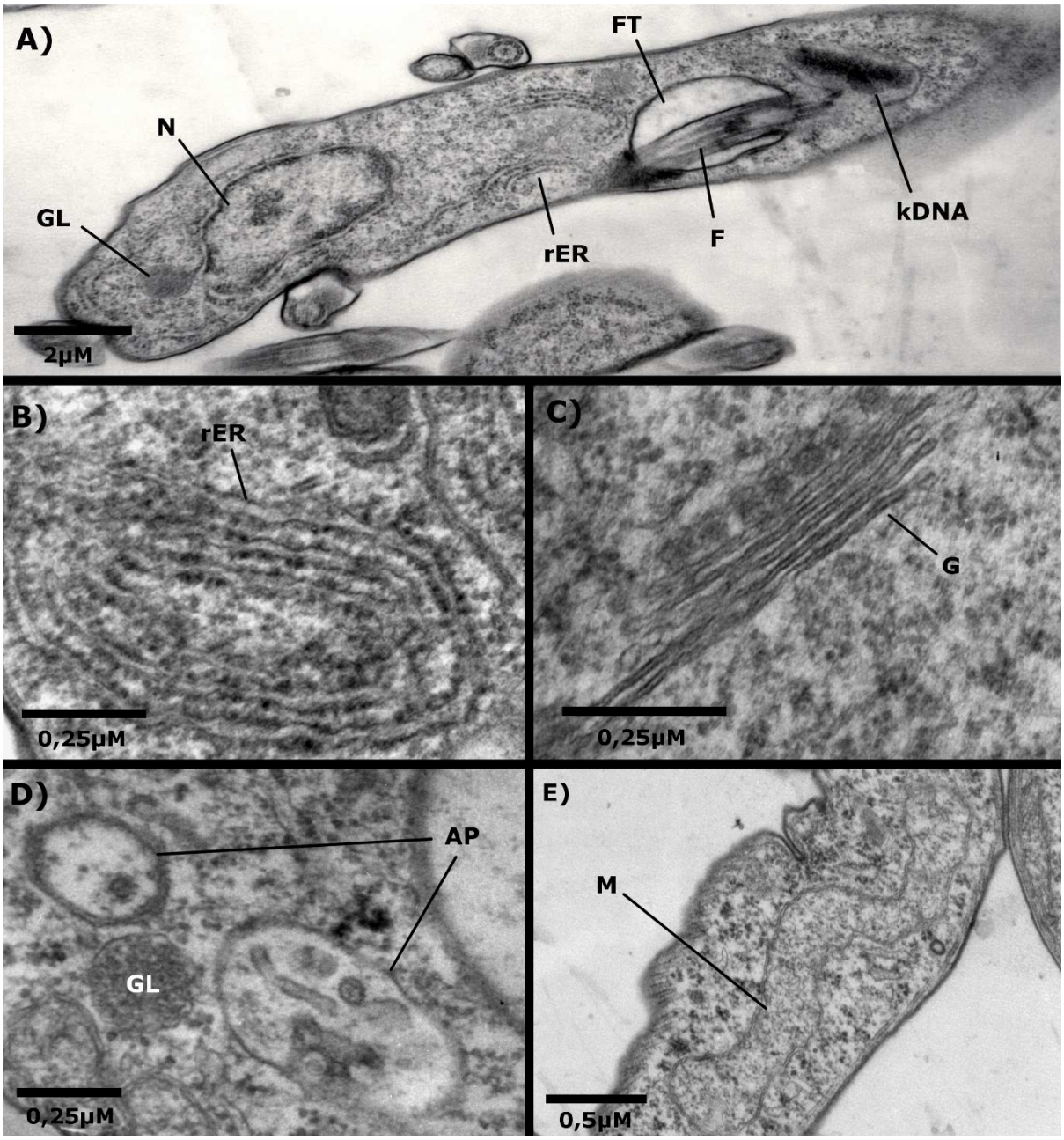
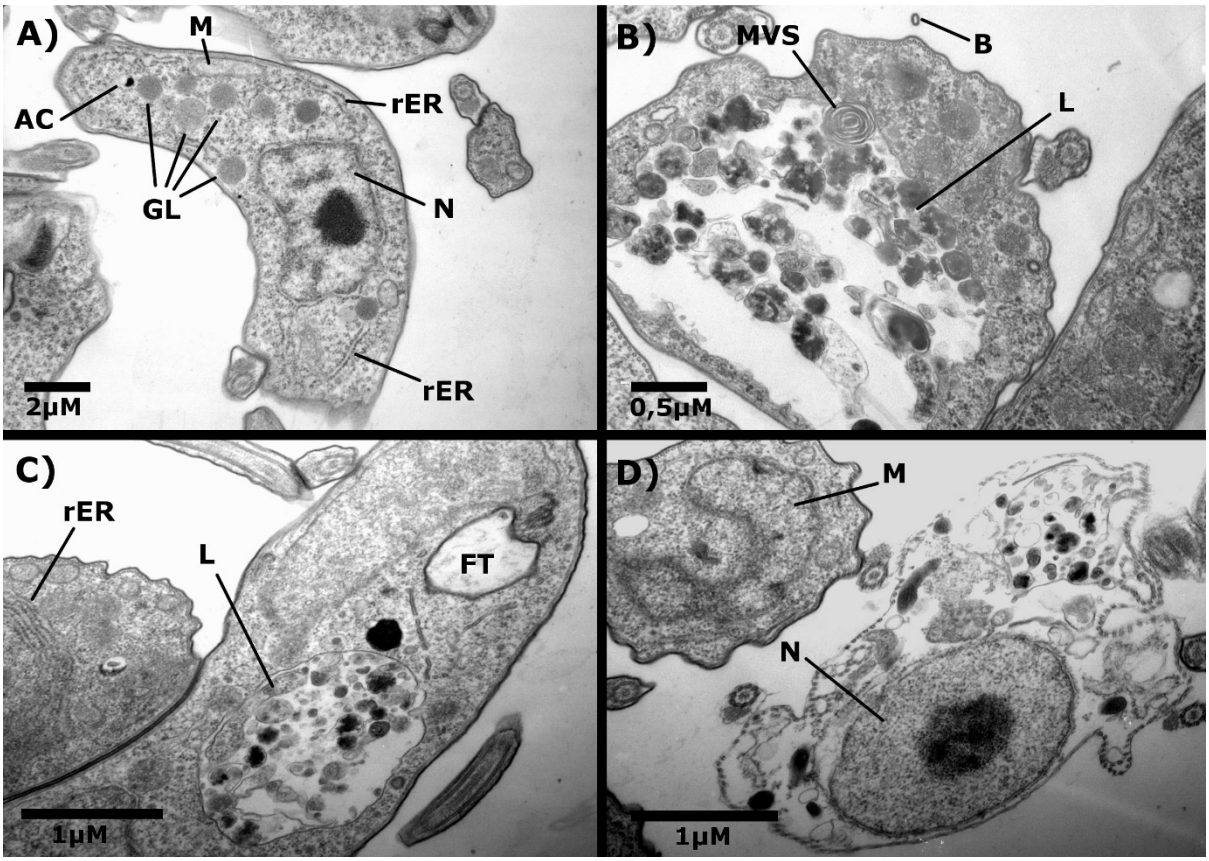


Figure 2. Ultrastructural alterations (TEM) in bloodstream forms of *T. brucei* treated with clomipramine hydrochloride (6 µM). Parasites were treated for 24 h. (A) untreated parasites; (B-D) clomipramine-treated parasites showed B) enlarged rough endoplasmatic reticulum (rER), C) increased Golgi apparatus (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.



295
296
297 **Figure 3. Ultrastructural alterations (TEM) in bloodstream forms of *T. brucei* treated**
298 **with clomipramine hydrochloride (6 µM).** Parasites were treated for 24 h. (A) untreated
299 parasites; (B-D) Clomipramine-treated parasites. B) and D) Lysosomes filled with plenty of
300 cellular material of organelle origin, D) dead cell after clomipramine treatment. **AC**=
301 acidocalcisome, **B** = blebbing, **FT** = flagellar pocket, **GL** = glycosome, **L** = lysosome, **M** =
302 mitochondrion, **MVS** = multivesicular structure, **N** = nucleus, **rER** = rough endoplasmatic
303 reticulum.
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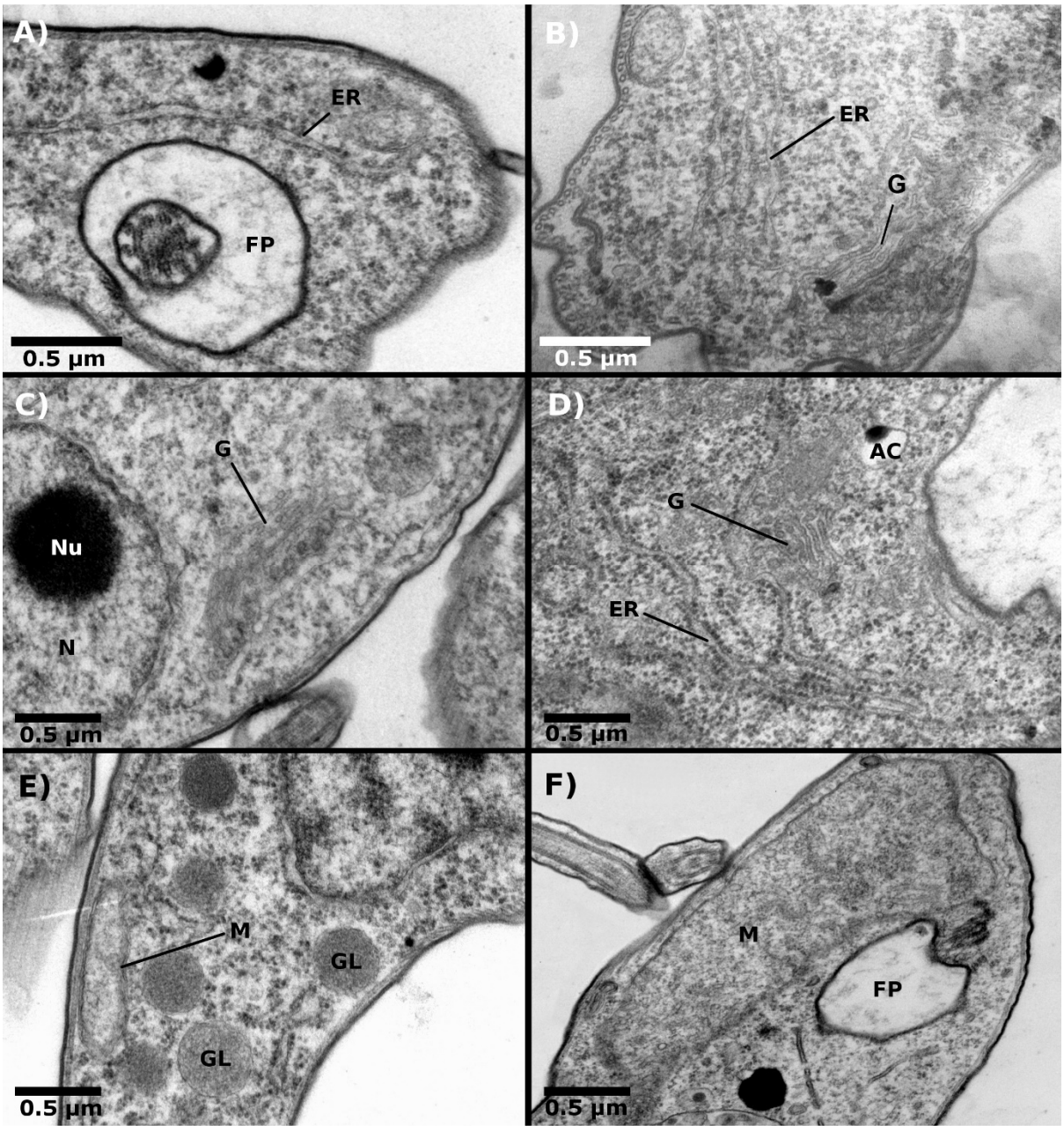

Figure 4. Ultrastructural alterations (TEM) in bloodstream forms of *T. brucei* treated with clomipramine hydrochloride (6 µM). Parasites were treated for 24 h. (A, C, E) = untreated parasites; (B, D, F) = Clomipramine-treated parasites. (B, D) increase of rER membranes and Golgi stacks as compared to 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 endoplasmatic reticulum.

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 Figure 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%.

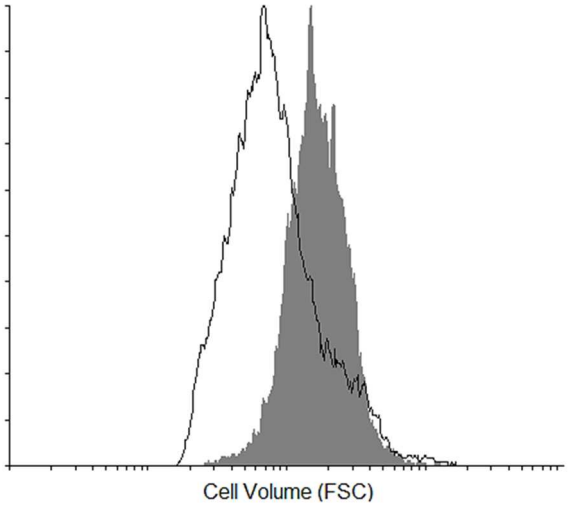

Figure 5. Cell volume alteration of *T. brucei* treated with clomipramine hydrochloride for 24 h. The histograms assessed by flow cytometry show the relationship between the numbers of cells (counts) and Forward Scatter (FSC) considered as a function of cell volume. The gray-filled area represents untreated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

Mitochondrial-membrane potential

Since our results suggested that apoptosis may be involved in clomipramine-induced cell death, we also measured the mitochondrial-membrane potential (ΔΨm). Our results show an intense decrease in TMRE fluorescence inside the mitochondrion after clomipramine treatment, which is directly related to the loss of ΔΨm (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.

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).

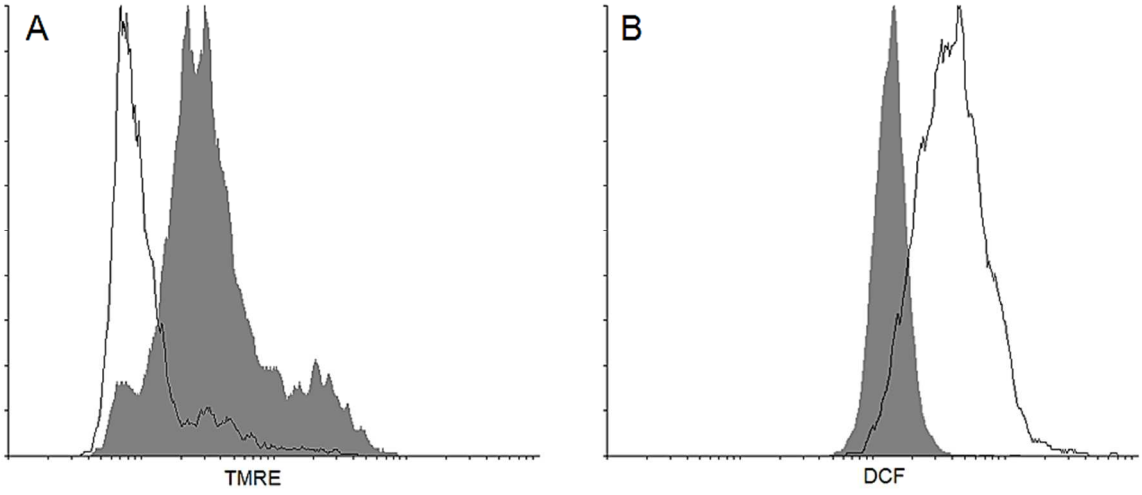

Figure 6. Evaluation of mitochondrial-membrane potential (ΔΨ) and measurement of intracellular reactive oxygen species (ROS) level in *T. brucei* bloodstream forms after 24 h clomipramine hydrochloride treatment. (A) Parasites were stained with the fluorescent probe TMRE (2.5 nM) and analyzed by flow 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.

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).

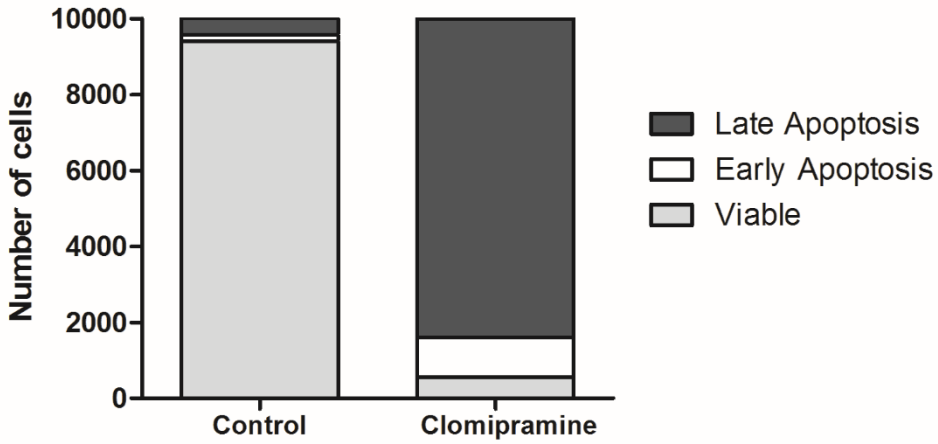

Figure 7. Exposure of phosphatidylserine in *T. brucei* following a 24 h treatment with clomipramine hydrochloride. Parasites were treated with clomipramine (6 µM) for 24 h and analyzed by flow cytometry and classified regarding the cell death profile according to differential co-staining with annexin-FITC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

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). 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.

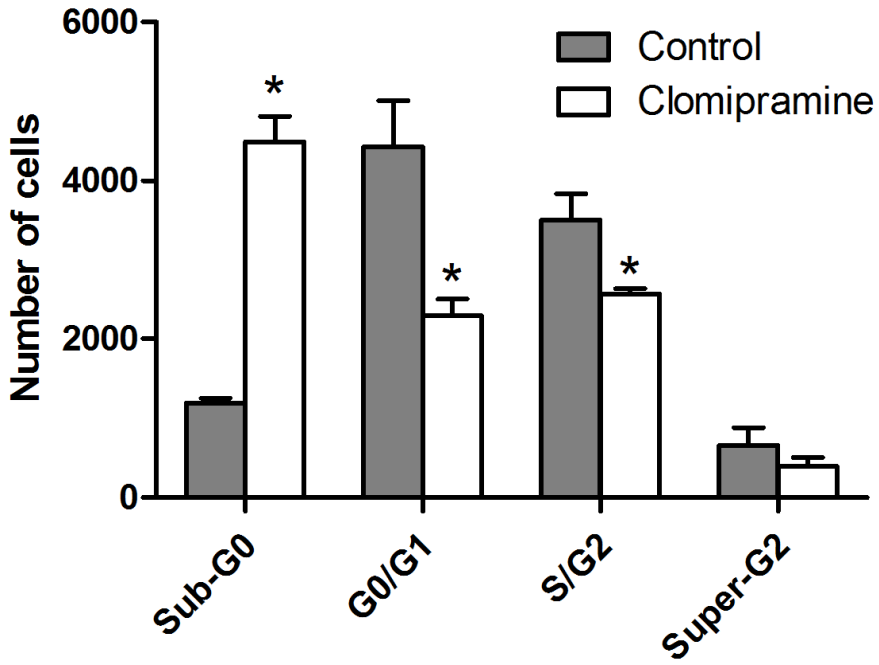

Figure 8. Cell cycle analysis of *T. brucei* bloodstream forms treated with clomipramine hydrochloride (6 µM) for 24 h. Parasites after treatment were permeabilized, stained with PI (10 µg/mL) and analyzed by flow cytometry. The bars show the median ± standard deviation of number of parasites found in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

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 Mat. and Meth. section) for mean fluorescence in pre-treated parasites changed to a value of -0.11, 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 IV values were about to 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.

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 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 anti-neoplastic 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 *Trypanosoma 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 and Comini, 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 (Fig. 2 and 3). This substantial increase in volume, can be understood as a physiological response to the 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., 2016). 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 Figure 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., 2009). 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 PI3K 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., 2009), however the signaling pathway by which this cross-talking takes place on the parasite is still poorly understood.

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.

FUNDING

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COMPETING INTERESTS

All the authors declare no competing interests.

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FULL PAPER 2

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The antidepressant Clomipramine induces programmed cell death in *Leishmania amazonensis* through a mitochondrial pathway

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ABSTRACT

Despite many efforts, the therapies currently available for the treatment of Leishmaniasis are not fully efficient. In an attempt to find new medicaments, drug repurposing arises as a promising strategy. Here we present data that supports further investigations on the use of the antidepressant clomipramine against *Leishmania amazonensis.* The drug showed selective activity at micromolar range against both the intracellular and extracellular parasite forms and additional stimulation of NO production in host macrophages. Regarding its mechanism of action, clomipramine induces severe mitochondrial depolarization, which coupled with the inhibition of trypanothione reductase end up inducing strong oxidative stress. Consequent effects observed in promastigotes includes lipoperoxidation, plasma membrane permeabilization and apoptotic signs (i. e. DNA fragmentation, phosphatidylserine exposition, cell shrinking, etc). Additionally, we performed also the isolation of intracellular amastigotes and verified similar mechanism of action as described for promastigotes, but coupled with energetic stress shown by the reduction in ATP levels. Such differences might be explained by metabolic particularities of each parasitic form. Ultrastructural alterations on the endomembrane system and formation of autophagic structures were also observed, possibly as an adaptive response front of the strict oxidative stress. Taken together, our results supports that clomipramine interfere on the redox metabolism of *Leishmania amazonensis* and, although the responses in order to rescue cell homeostasis, parasites terminates suffering programmed cell death.

KEYWORDS: *Leishmaniasis; Repurposing; Repositioning; Apoptosis; Intracellular amastigotes isolation; Autophagy*

INTRODUCTION

Leishmania is a proeminent genus of pathogenic protozoan that can resides and multiplicate inside one of the front line agent of the cellular immune system, the macrophages [1,2]. These intracellular protozoa present a complex digenetic life cycle, requiring a susceptible vertebrate host and a permissive insect vector, in general phlebotomines, which allow their transmission [3]. Leishmaniasis, the group of diseases caused by these parasites, comprise a severe public health problem in many countries around the globe, affecting mainly populations in regions of social and economic vulnerability [4].

The chemotherapy currently available for the treatment of leishmaniasis is not completely efficient, although the drugs have proven leishmanicidal activity there are severe toxicity problems, emergence of resistant strains and a complex protocol of administration. Undoubtedly, the liposomal formulation of amphotericin B (L-AMB) is the best option currently available for leishmaniasis treatment, presenting the highest therapeutic efficacy and the most favorable safety, but with limited due to its high costs [5]. Considering the presented limitations, several approaches on drug discovery have been used in order to find more effective and less toxic medicaments, including natural products isolation and screening of huge libraries of synthetic compounds. Nevertheless, despite the increasing investments on drug discovery on the last decades, these traditional approaches has not much often succeed on the development of novel and more efficient therapeutic alternatives [6]. On the pursuit of new alternatives, the search of novel uses for already approved drugs, known as drug repurposing, arises as a cheaper and faster approach to find new treatments for these parasitic diseases [7,8].

Clomipramine is a tryciclic antidepressant currently used in clinical on the treatment of psychiatric disorders, indicated for the management of obsessive-compulsive disorder and presenting a mechanism of action based on the inhibition of serotonin reuptake [9]. By employing the repurposing approach, Clomipramine was found to have potential activity on

the treatment of trypanosomiasis, inhibiting the proliferation of both *Trypanosoma brucei* [10] and *Trypanosoma cruzi* [11,12]. The molecular mechanism by which Clomipramine acts in trypanosomatids involves the competitive inhibition of Trypanothione reductase (TR), an enzyme that plays a key role on the unique redox metabolism of these parasites [13].

On the present work, we evaluate the activity of the tricyclic antidepressant Clomipramine against *Leishmania amazonensis*. Additionally, we describe the biochemical and ultrastructural alterations involved on the mechanism of action of Clomipramine in the parasite.

MATERIALS AND METHODS

Chemicals

Dimethyl sulfoxide (DMSO), folic acid, hemin, thiazolyl blue tetrazolium bromide (MTT), actinomycin D, antimycin A, carbonyl cyanide m-chlorophenylhydrazone (CCCP), camptothecin (Camp), potassium cyanide (KCN), nile red, *N*-acetyl-L-cysteine (NAC), digitonin, lipopolysaccharides (LPS) and monodansylcadaverine (MDC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Brain heart infusion (BHI) was acquired from Beckton Dickinson (Sparks, MD, USA). Fetal bovine serum (FBS), RPMI-1640 medium, and Giemsa were obtained from Invitrogen (Grand Island, NY, USA). Annexin V fluorescein isothiocyanate (FITC) conjugate, the APO bromodeoxyuridine (BrdU) terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay kit, 2',7'-dichlorofluorescin diacetate (H_2DCFDA); 4-amino-5-methylamino-2',7'-difluoro fluorescein diacetate (DAF-FM), Amplex red hydrogen peroxide/peroxidase assay kit and tetramethylrhodamine (TMRE) were obtained from Invitrogen (Eugene, OR, USA). Cell titer-

glo luminescent cell viability assay was obtained from Promega ® (Madison, USA). All of the other reagents were of analytical grade.

Compound

Clomipramine hydrochloride (Clomi) was purchased from Sigma-Aldrich (St. Louis, MI, USA). The drug was diluted in DMSO before each experiment and the final concentration of diluent in all assays never exceeded 0.5%, which had no influence on the parasites in control experiments.

Parasites and Mammalian cells

The experiments were performed with *Leishmania amazonensis* (strain WHOM/BR/75/JOSEFA) originally isolated from a patient with diffuse cutaneous leishmaniasis by C.A. Cuba-Cuba (Universidade de Brasília, Brazil). Promastigote forms were cultivated in Warren medium (brain heart infusion, hemin, and folic acid; pH 7.0) supplemented with 10% FBS and maintained at 25 ºC. Macrophages (J774A1) were maintained in RPMI-1640 medium (pH 7.2), added with sodium bicarbonate and L-glutamine, supplemented with 10% FBS and maintained at 37 ºC in a 5% CO_2 atmosphere. Intracellular amastigotes forms were obtained from macrophages infected with promastigotes, cultivated at the conditions established for macrophages but at 34 ºC.

Antiproliferative activity against promastigotes of *L. amazonensis*

Parasites harvested at exponential growth phase (1 x 10^6 promastigotes/mL) were incubated in the absence or presence of different concentrations of Clomi, for 72 h. After that,

parasites growth was estimated by counting the parasites in a Neubauer haemocytometer, IC_{50} and IC_{90} values (concentration that inhibited 50% and 90% of the parasite growth) were determined. The results were expressed as percentage of growth inhibition compared with control cultures.

Antiproliferative activity against intracellular amastigotes of *L. amazonensis*

To assess the activity against intracellular parasites, JJ74-A1 macrophages were infected with promastigotes (10 parasites per host cell), and incubated for 24 h at 34 ºC with 5% CO_2 atmosphere. After this, the infected macrophages were treated with different concentrations of Clomi and incubated for 48 h, following fixation in methanol and Giemsa staining. The numbers of infected cells and amastigotes were counted in 100 cells. The infection indexes (nº of amastigotes per cell x percentage of cells infected / total nº of cells) were determined and calculated the IC_{50} and IC_{90}. All the results were expressed as the means of three independent experiments.

Cytotoxicity in J774-A1 macrophages

Macrophages were cultured ($5x10^5$) cells/ml in RPMI-1640 medium supplemented with 10% FBS in 96-well microplates at 37 °C in a 5% CO_2 atmosphere. After 24 h, cells were treated with different concentrations of Clomi and incubated for additional 48 h. The microplates were then washed with PBS, and 50 µl of MTT solution (2 mg/ml) was added to each well and the plate was incubated for 4 h at 37 °C protected from light. After de incubation 150 µl DMSO was added to each well and the absorbance read in a Bio-Tek Power Wave XS spectrophotometer at 570 nm. The MTT assay is based on the conversion of water-soluble MTT to an insoluble formazan precipitate by viable mitochondria. The cytotoxicity

concentration for 50% of the cells (CC_{50}) was determined. The selectivity index (SI) was calculated as CC_{50}/IC_{50}.

Isolation of intracellular amastigote forms

In order to study the mechanism of action of Clomi direct in intracellular amastigote we established a protocol for isolation of these parasites. For that, JJ74-A1 macrophages were infected with promastigotes (10 parasites per host cell), and incubated for 48 h at 34 ºC with 5% CO_2 atmosphere. The infected macrophages were treated with Clomi at 15 and 30 µM, and then incubated for additional 24 h. After treatment, infected macrophages were removed using a cell-scraper and lysed by aspiration and extrusion with a syringe and a needle (30 G x 1/2 inches BD PrecisionGlide™ - Canada). Amastigotes were separated from unlysed macrophages and debris by differential centrifugation at 1000 rpm for 5 min, and collected on the supernatant. The purity of the isolated fraction was assured under microscope examination, the number of amastigotes quantified in a Neubauer hemocytometer and the suspensions adjusted to 1 x 10^6 parasites/ml.

Production of nitric oxide (NO) of macrophages

Briefly, macrophages were plated in 96-well black plates and incubated at 34 ºC with 5% CO_2 atmosphere, for 48 h. Cells were then treated with Clomi (15 and 30 µM), and incubated in the same conditions for 24 h. After treatment, cells were loaded with DAF-FM diacetate (1 µM), incubated for 30 min at room temperature, washed, resuspended in phosphate-buffered saline (PBS) and incubated for additional 15 min. The fluorescence was measured in spectrofluorometer (Victor X3; PerkinElmer) at λ_{ex} = 495 nm and λ_{em} = 515 nm.

H_2O_2 (4 mM) and LPS (1 µg/ml) were used as a positive control. In parallel, we performed the pretreatment of the same groups with NAC (5 mM) for 3 h.

Morphological and ultrastructural analysis

In order to assess the morphological and ultrastructural alterations induced by clomipramine, parasites were analyzed by Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM). Promastigotes were treated with Clomi (8 and 22 µM) for 72 h and prepared for SEM. To visualize alterations on the intracellular form of *L. amazonensis*, macrophages were infected with promastigotes for 48 h, treated for 24 h with the drug at 15 and 30 µM, and then proceed to TEM preparation. After treatment, both parasitic forms were washed with PBS, fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at 4°C. For TEM analysis samples infected macrophages were post-fixed in a solution containing 1% osmium tetroxide (OsO_4), 0.8% potassium ferrocyanide, and 10 mM $CaCl_2$ in 0.1 M cacodylate buffer, dehydrated in increasing acetone gradient and embedded in Epon® resin 72 h at 60°C. Ultrathin sections were obtained, stained with uranyl acetate and lead citrate and examined on a JEOL JM 1400 TEM. For SEM, promastigotes were placed on a glass specimen support with poly-L-lysine, dehydrated in graded ethanol, critical-point dried in CO_2, coated with gold and observed on a FEI Quanta 250 FEG Scanning Electron Microscope.

Evaluation of the mechanism of action of Clomi in *L. amazonensis* promastigotes and amastigotes

Aiming the elucidation of the mechanism of action and the cellular alterations induced by clomi treatment of *L. amazonensis*, we performed a set of spectrometric experiments. As a general procedure, promastigotes (1×10^6 cells/ml) were treated with clomi at 8 and 22 µM for

24 h, and intracellular amastigotes (1×10^6 cells/ml) isolated from macrophages previously incubated with clomi 15 and 30 µM for 24 h, in the same cultivation conditions as previously described. In parallel, for the assays involving promastigotes, before the drug treatment, we also performed a pre-incubation of half of the parasites with the antioxidant *N*-acetyl cysteine (200 µM NAC) for 3 h, in order to identify a possible ROS-involvement on some parameters evaluated. The number of parasites was determined, normalized and the staining procedure for each assay, conducted as described of the next sub-sections. All the fluorometric and luminometric measurements were performed in a plate multireader (Victor X3; PerkinElmer®), the specifics wavelength of excitation (λ_{ex}) and emission (λ_{em}) are presented for each assay. Experiments were repeated at least three time independently.

Determination of mitochondrial membrane potential ($\Delta\Psi m$)

Since mitochondrion is an important drug target into the parasite, we applied the cationic probe TMRE in order to evaluate the inner mitochondrial membrane potential. For this purpose, promastigotes previously treated were washed in PBS and incubated with 25 nM TMRE for 30 min in the dark. After incubation, the parasites were washed again to remove non-internalized TMRE, resuspended in PBS and distributed in a 96-well black plate. The reading was performed in a spectrofluorometer at λ_{ex} = 540 nm and λ_{em} = 595 nm. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; 100 µM) was used as a positive control.

Measurement of intracellular ATP levels

Still considering the influence of the drug on the energetic metabolism of the parasites we measured the cellular ATP levels. This quantification was performed by the Cell Titer-Glo Luminescent Cell Viability Assay. After treatment, parasites were collected, washed and resuspended in PBS buffer. In a white 96-wells plate an equal volume of CellTiter-Glo reagent (50 µl) was added, mixed with an aliquot of each sample (50 µl) and incubated for 10 min. The

luminescence was measured in a microplate reader (VICTOR X3, PerkinElmer). Potassium cyanide (KCN; 500 µM) was used as a positive control.

Measurement of total ROS levels

Regarding the influence of clomi on redox metabolism of *Leishmania* the level of ROS was determined by the probe H_2DCFDA. After clomi treatment, parasites were collected, washed and resuspended in PBS buffer and incubated with H_2DCFDA (10 µM) for 45 min in the dark. The samples were added in a 96-well black plate and the fluorescence was spectrofluorometrically measured at λ_{ex} = 488 nm and λ_{em} = 530 nm. As a positive control, a group of cells were treated also for 24h with H_2O_2 (4 mM).

Measurement of H_2O_2 levels

In addition, the quantification of H_2O_2 with the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit was also performed. After treatment with clomi, the parasites were collected, washed and resuspended in PBS buffer (5 mM succinate) and processed according to the manufacturer's manual. Then the samples were added in a 96-well black plate and the fluorescence was measured in spectrofluorometer (Victor X3; PerkinElmer) at λ_{ex} = 571 nm and λ_{em} = 585 nm. H_2O_2 (4 mM) was used as a positive control.

Measurement of mitochondrial $O_2^{\bullet-}$ levels

The generation of mitochondrial $O_2^{\bullet-}$ was detected by the MitoSOX Red mitochondrial superoxide assay kit. The experiments were performed according to the manufacturer recommendations, with short adjustments. Briefly, promastigotes treated or not as previously described were washed and resuspended in Krebs-Henseleit buffer (KH; 15 mM $NaHCO_3$, 5 mM KCl, 120 mM NaCl, 0.7 mM Na_2HPO_4, and 1.5 mM NaH_2PO_4; pH 7.3) and incubated with MitoSOX reagent (5 µM) for 20 min in the dark at 25 °C. After incubation, the parasites

were washed and resuspended again in KH buffer. The reading was performed at λ_{ex} = 510 nm and λ_{em} = 580 nm. Antimycin A (AA; 10 µM) was used as a positive control.

Measurement of NO levels

Quantification of NO was determined by DAF-FM diacetate. After treatment with Clomi, promastigotes were collected, washed, resuspended in PBS buffer and incubated with DAF-FM diacetate (1 µM) for 30 min in the dark at RT. After incubation, the parasites were washed/resuspended in PBS buffer and incubated for an additional 15 min. Then the samples were added in a 96-well black plate and the reading was performed in a spectrofluorometer (Victor X3; PerkinElmer) at λ_{ex} = 495 nm and λ_{em} = 515 nm. H_2O_2 (4 mM) was used as a positive control.

Measurement of reduced thiol levels

Considering the antioxidant defense of the kinetoplastids relies mainly on trypanothione, a thiol-based molecule, we quantified the level of reduced thiol by the DTNB method, as previously performed (Bidóia *et al*., 2013). Clomi treated promastigotes ($1x10^7$ cells/ml) were collected, centrifuged, resuspended in Tris-HCl buffer (100 mM; pH 2.5) and sonicated. The samples were then centrifuged and the supernatant collected. In 96-wells plates samples were incubated with DTNB (1 mM) in PBS. The reading of absorbance was performed on a microplate reader at 412 nm (BIO-TEK Power Wave XS spectrophotometer). H_2O_2 (4 mM) was used as a positive control.

Lipid peroxidation evaluation

Bearing in mind that ROS may led to alterations on cell lipids structure and distribution, we measured lipid peroxidation and accumulation of lipid droplets by using the probes DPPP and Nile Red. For that, treated promastigotes were collected, washed and resuspended in PBS buffer and incubated with DPPP (5 µM) or Nile red (50 µM) in the dark at RT, for 15 min or

30 min respectively. Samples were added in a 96-well black plate and the fluorometric readings performed at $\lambda_{ex/em}$ = 355/460 nm for DPPP and $\lambda_{ex/em}$ = 385/535 nm for Nile red. In both assays H_2O_2 (4 mM) was used as a positive control.

Analysis of cell membrane integrity

Since lipids are the main component of cell membrane, we evaluated the integrity of this barrier by using the probe PI, a non-permeable marker that emits high fluorescence when intercalating nucleic acids. After treatment with Clomi, the parasites were collected, washed and resuspended in PBS buffer and incubated with PI (0.2 µg/ml) for 5 min protected from light at RT. Then the samples were added in a 96-well black plate and the reading performed in a spectrofluorometer (Victor X3; PerkinElmer) at 535/617 nm. The detergent digitonin (40 µM) was used as a positive control.

Detection of DNA fragmentation by TUNEL assay

In order to determine DNA fragmentation, treated parasites were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 for 10 min and then incubated with a TdT reaction mixture containing BrdUTP for 2 h at 37°C according to the manufacturer's instructions with minor modifications (APO-BrdU TUNEL Assay Kit, with Alexa Fluor 488 Anti-BrdU). Cells linked with BrdU were detected using a green-fluorescent Alexa Fluor 488 dye-labeled anti-BrdU antibody for 30 min. Fluorescence was quantified in a spectrofluorometer at $\lambda_{ex/em}$ = 485/520 nm. Camptothecin (10 µM) was used as a positive control.

Detection of exposure of phosphatidylserine

Exposure of phosphatidylserine (PS) was detected by the probe annexin-V/FITC. After treatment with Clomi, promastigotes were collected, washed in PBS, resuspended in binding buffer (140 mM NaCl, 5 mM $CaCl_2$, and 10 mM HEPES-Na, pH 7.4) buffer and incubated

with anenxin-V/FITC for 15 min in the dark at RT. Samples were the added in a 96-well black plate and the reading was performed in a spectrofluorometer at $\lambda_{ex/em}$ =494/518 nm. The apoptosis inducer Camptothecin (10 µM) was used as a positive control.

Caspase 3/7-like activity

In addition to the others apoptotic parameters evaluated, the quantification of caspase-like active was determined by the EnzChek Caspase-3 Assay Kit #1 Z-DEVD-AMC Substrate. Treated promastigotes were collected, washed and resuspended in PBS buffer and stained according to the manufacturer's manual. Then the samples were added in a 96-well black plate and the reading was performed in a spectrofluorometer at $\lambda_{ex/em}$ = 342/441 nm. Camptothecin (10 µM) was used as a positive control. In order to corroborate the response observed, similar groups were also pre-incubated with the apoptosis inhibitor Ac-DEVD-CHO.

Autophagic vacuoles detection

Quantification of autophagic vacuoles was determined using the MDC. After treatment with clomi, promastigotes and amastigotes were collected, washed and resuspended in PBS buffer and incubated with MDC (0.05 mM) for 1 h in the dark at RT. Then the samples were added in a 96-well black plate and the reading was performed in a spectrofluorometer (Victor X3; PerkinElmer) at λ_{ex} = 335 nm and λ_{em} = 518 nm. As positive control for autophagy we used 7-day old cultures of promastigotes, which naturally go into autophagy. In addition, for promastigotes we also performed in parallel the pretreatment of a group with the wortmannin (WT; 0.5 µM), a potent inhibitor of PI3 kinase and consequently of autophagic vacuoles formation (Blommaart et al., 1997).

Statistical analysis

All quantitative experiments were conducted in at least three independent experiments in duplicate, data are expressed as means and standard error. The statistical analyses were performed using the GraphPad Prism 5.0 software (San Diego, CA, USA). The data were analyzed using one-way or two-way analysis of variance (ANOVA) when appropriate, and Bonferroni *post hoc* test was used to compare means. Values of $p \leq 0.05$ were considered statistically significant.

RESULTS

Clomipramine selectively inhibits the proliferation of *L. amazonensis*

In order to analyze the potential of the repurposing of the antidepressant clomi on the treatment of leishmaniasis we performed antiproliferative assays against both promastigote and amastigote forms of *L. amazonensis.* Our results indicate clomi as a selective inhibitor of the extracellular and intracellular forms of the parasite. Against promastigotes clomi expressed IC_{50} of 8.31 ± 3.29 µM and IC_{90} equal to 21.58 ± 3.44 µM. The drug was also able to inhibit the proliferation of the intracellular form of the parasite presenting IC_{50} equal to 15.45 ± 4.92 µM and IC_{90} of 31.38 ± 3.27 µM. Regarding the cytotoxicity the drug exhibits a safe profile, inhibiting 50% of the macrophages cell growth just at 181.22 ± 8.04 µM. Taken together these results indicate great selective indexes for clomi, with values of 11.72 in amastigotes and 21.81 in promastigotes.

Production of nitric oxide (NO) by macrophages is stimulated by Clomipramine

In order to verify the effect of Clomi directly on the macrophages, we performed the quantification of NO produced by these cells after treatment, using the probe DAF-FM diacetate. Our findings indicate that the tryciclic antidepressant is able to induce the increase

in intracellular NO levels in macrophages in a dose dependent way, increasing in more than 50% the fluorescence values after 30 µM treatment (Fig. 1). As expected, the well-known macrophages activator, LPS, led to pronounced increase in NO generation.

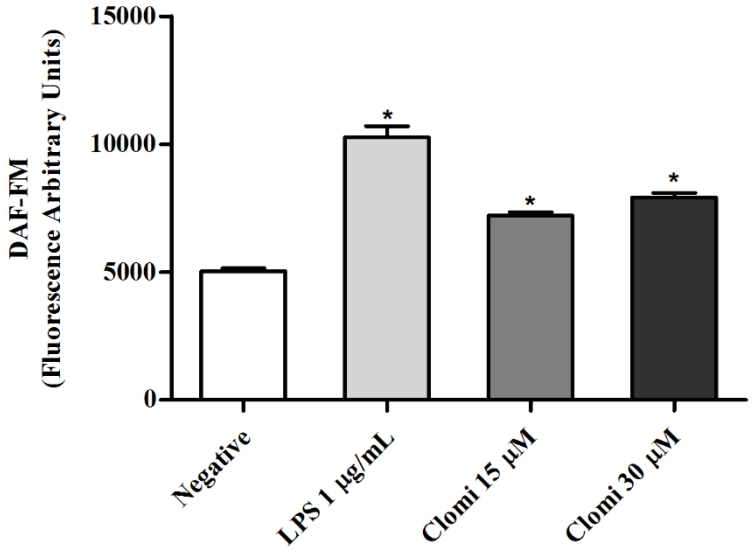

Figure 1. Production of Nitric Oxide (NO) by J774.A1 macrophages after 24 h treatment with clomi. Levels of NO were measured in a spectrofluorimeter using the fluorescent probe DAF-FM. Data are expressed as means ± SE of at least three independent experiments. Lipopolysaccharide (LPS) was used as an activator for macrophages. * $p \leq 0.05$ compared to untreated control.

Clomipramine induces morphological and ultrastructural alterations in *L. amazonensis*

Once determined the inhibitory concentrations of clomi, we performed SEM analysis of the promastigotes in order to determine the morphological alterations derived from the treatment. Untreated cells showed normal elongated body with a prominent flagellum, exhibiting smooth

and intact cell surface (Fig 2A). In contrast, parasites treated with Clomi presented severe alterations in cell shape (Fig. 2), with wrinkled cell surface, reduction of body size and distortions of the flagellum, but with maintenance of cell membrane integrity. Interestingly we also observed accumulation of vesicles on the surface of flagellar pocket (Fig. 2C) of treated promastigotes. In what refers to ultrastructural alterations, *L. amazonensis* was also strongly affected by Clomi treatment.

For a complete ultrastructural analysis treated and control amastigotes were focused on large parasitophorous vacuoles of infected macrophages. One remarked alteration denoted on treated parasites is the flagellar pockets full of vesicles, possibly indicating intense exocityc activity (Fig. 3B; D; F). Another prominent consequence of the treatment is the large multivesicular vacuoles spread around the cytosol, filled with electron-dense cargo and full of membranous arrangements (Fig. 3C – F). Companied by this abundance of vesicles in the cytoplasm, the endomembrane system organelles also appears clearly augmented in treated amastigotes, already at IC_{50} treatment. An increase in lipid inclusions bodies can also be denoted in Fig 3C, E and F. These referred alterations were already observed at Clomi IC_{50} treatments, but get even more evidence when IC_{90} was used. Additional feature observed on treated amastigotes include mitochondria swelling (Fig. 3E).

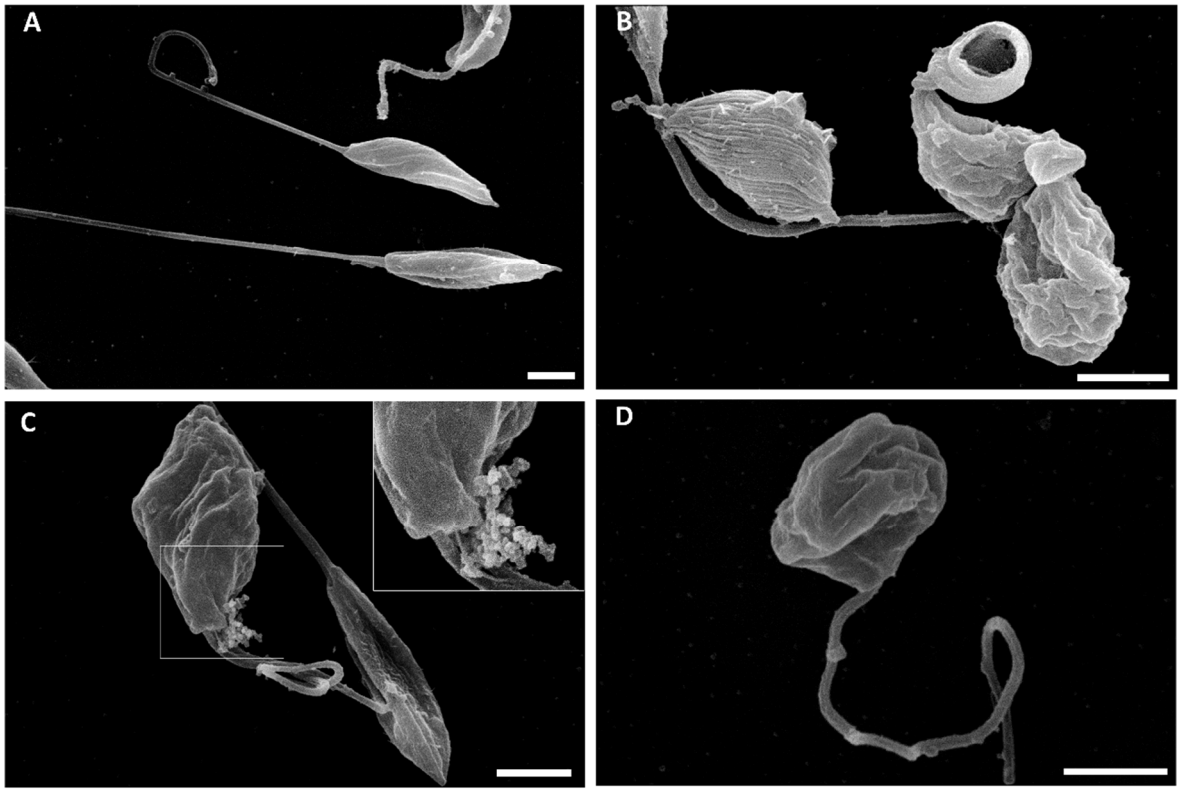

Figure 2. Morphological analysis (Scanning Electron Microscopy) of *L. amazonensis* promastigotes treated with clomi for 72 h. (A) Control parasites show a normal elongated body, exhibiting smooth and intact cell surface. (B) Promastigotes treated with the 8 µM presented a wrinkled cell surface, altered flagellum and cell size reduction. (C-D) After 22 µM treatment cells show the same alterations as previously described and additional accumulation of vesicles in the flagellar pocket region (detail in C) Bars = 2 µm

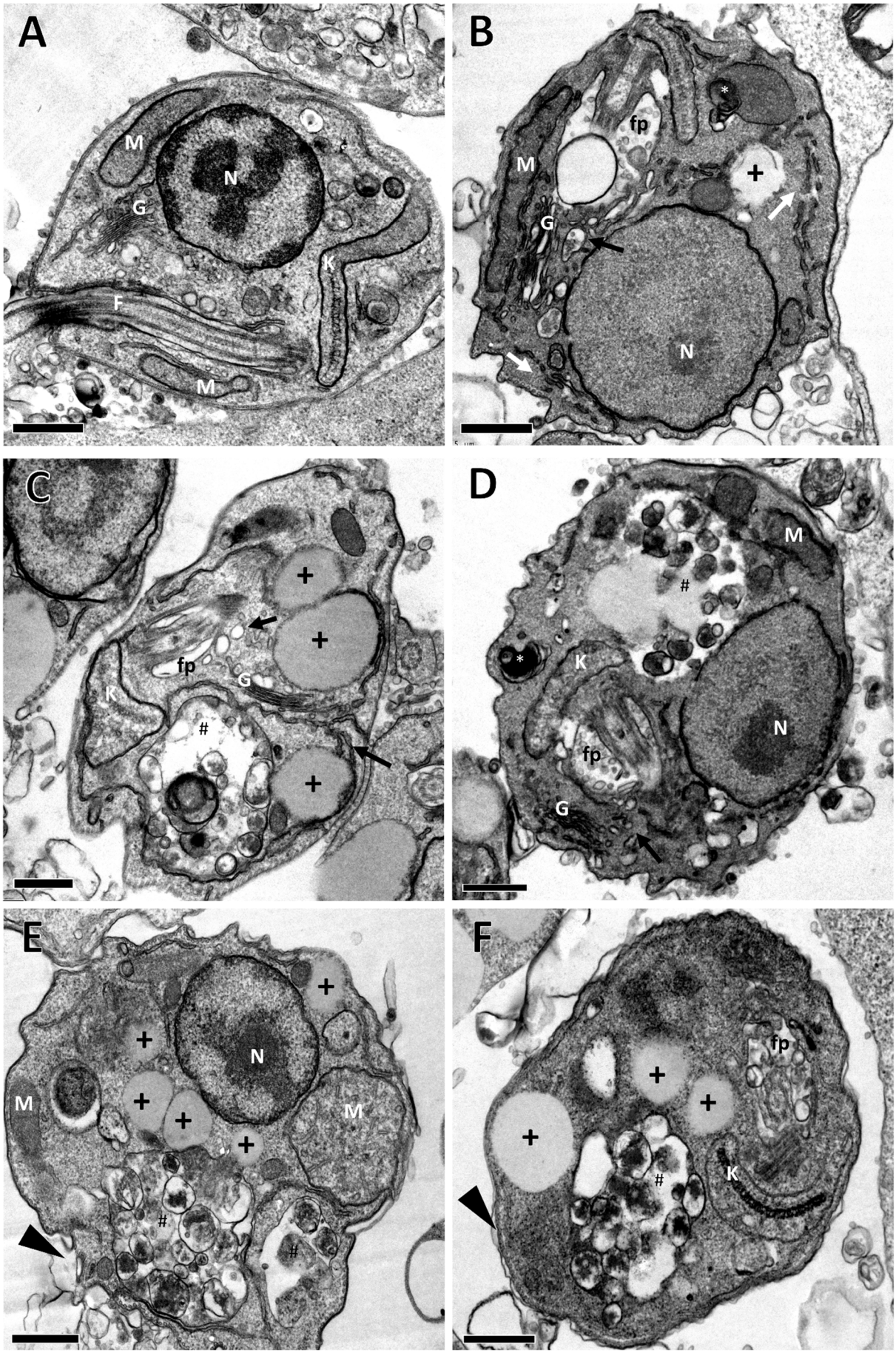
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Figure 3. Ultrastructural analysis (Transmission Electron Microscopy) of *L. amazonensis* amastigotes focused on vacuoles of J774.A1 macrophages, after 48 h clomi treatment. Control parasites (A) exhibited a normal ultrastructure with preserved organelles. Parasites treated with 15 µM (B-C) and 30 µM (D-F), present altered Golgi complex with enlarged cisternae, disassembled ER (white arrows), intense vesicles traffic (black arrows) and flagellar pocket full of cargo. Numerous lipid inclusions (+) can also be seen throughout the cytoplasm in all the treatments. Autophagy related structures, like double membrane autophagosomes, huge autolysosomes (#) and myelin-figures were very often observed. Plasma membrane shedding (black arrowhead) was verified on the most affected cells. N, nucleus; K, kinetoplast; F, flagellum; M, mitochondrion; G, Golgi complex; fp, flagellar pocket. Bars = 0.5 µm

Clomipramine induces oxidative stress in *Leishmania amazonensis*

The treatment with Clomi 8 µM was able to induces several alterations on the redox metabolism of promastigotes (Fig. 4A). On the pursuit of describing which reactive specie was specifically more abundant on the clomipramine-induced oxidative stress, we performed specific staining procedure. By amplex staining we verified that the amount of H_2O_2 formed almost doubled after the treatment at 8 µM Clomi (Fig. 4B). A different response was observed when mitochondrial superoxide (Fig. 4C) and nitric oxide (Fig. 4D) were measured, no statistically significant alteration was detected at the lower concentration tested, just at 22 µM the drug led to some response. The antioxidant NAC acted preventing the oxidative stress induced by Clomi, when previously applied, in most of the cases. Also related with the redox metabolism, the levels of reduced thiols were also affected by Clomi treatment (Fig. 5A), corroborating the inhibition of the enzyme Trypanothione reductase. Similarly, when intracellular amastigotes were treated we observed a clear augment on the amount of total ROS in the parasites (Fig. 7D) as well in the specific levels of H_2O_2 (Fig. 7C), clearly indicated by

increase in DCF and Amplex-Red fluorescence in isolated amastigotes treated by Clomi (15 and 30 µM).

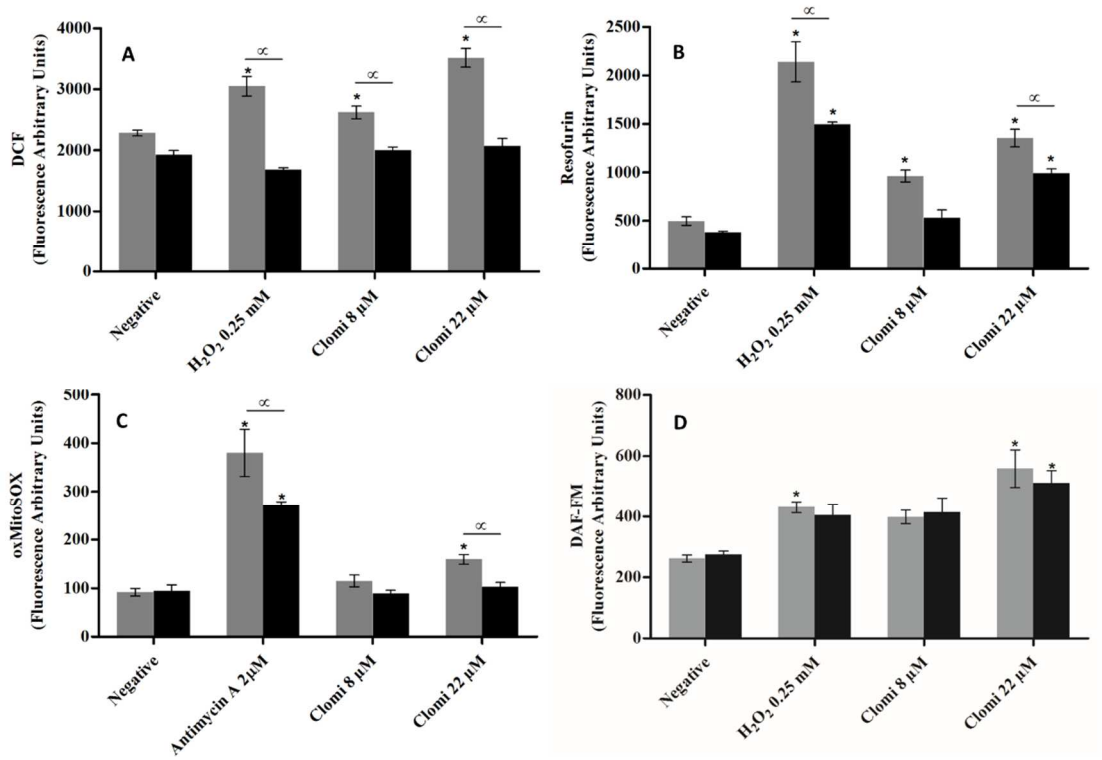

Figure 4. Measurement of the levels of Reactive Oxygen/Nitrogen species in *L. amazonensis* promastigotes treated for 24 h with Clomi (8 and 22 µM). H_2DCF-DA (A) evaluate total ROS, Amplex-Red (B) measure H_2O_2 production, (C) MitoSOX-red detect mitochondrial $O_2^{\cdot -}$ and (D) DAF-FM indicates the level of intracelullar NO. Gray bars were treated just with clomi, while black bars represent parasites pre-incubated with NAC 200 µM for 3 h before clomi addition. Antimycin A 2 µM and H_2O_2 0.25 mM were both used as oxidative stress inductors. * $p \leq 0.05$ compared to untreated control; α represents $p \leq 0.05$ for comparison between the pre-treated and the non-pre-treated with NAC.

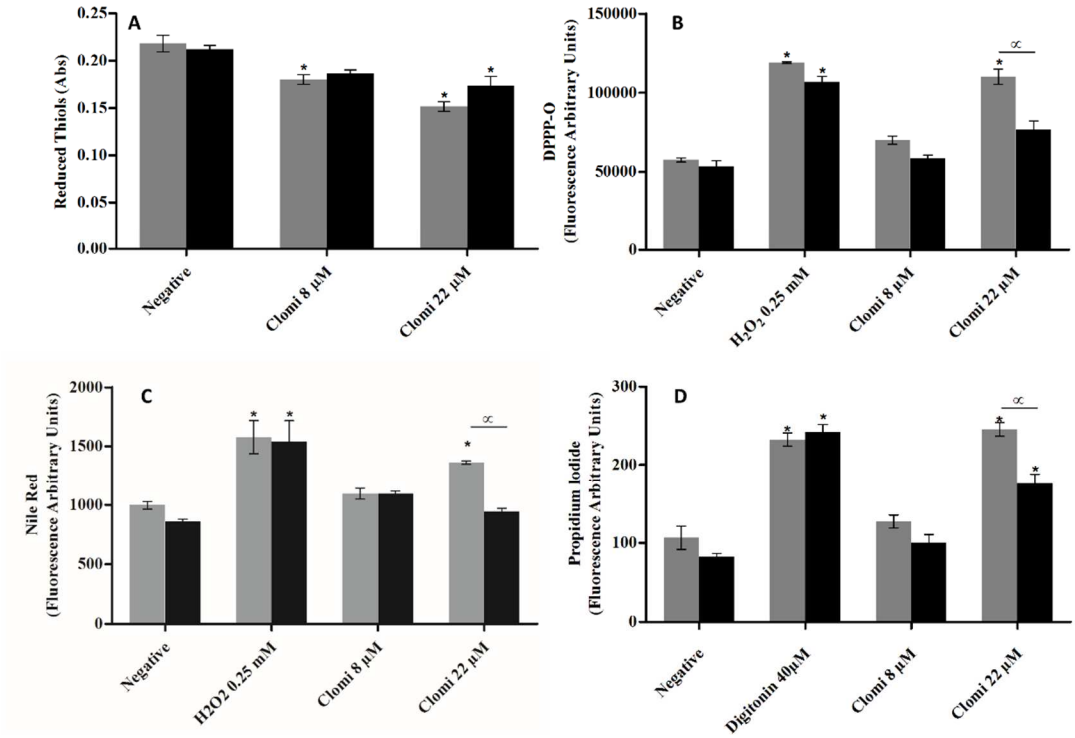

Figure 5. Evaluation of the levels of reduced thiols (A), lipoperoxidation (B), lipid droplets accumulation (C) and permeability of plasmatic membrane (D) in promastigotes of *L. amazonensis* treated for 24 h with clomi (8 and 22 µM). Gray bars represent parasites treated just with clomi, while black bars represent the ones pre-incubated with NAC 200 µM for 3 h before clomi addition. H_2O_2 0.25 mM was used as an oxidative stress inductor and the detergent digitonin (40 µM) as a membrane disruptor. * $p \leq 0.05$ compared to untreated control; α represents $p \leq 0.05$ for comparison between the pre-treated and the non-pre-treated with NAC for each treatment.

The oxidative stress end up affecting promastigotes cell membrane

The intense oxidative stress resultant from clomi treatment led to alterations on the integrity of cell membranes. Although no significant effect was observed at IC_{50} treatment, clomi at 22 µM induced an increase of lipid peroxidation on promastigotes (Fig. 5B) Similarly, the drug induced also accumulation of lipid bodies (Fig. 5C) and alterations on the cell membrane permeability (Fig. 5D) at the higher concentration tested. All these effects were at least partially inhibited when parasites were pretreated with NAC. On the other hand, in intracellular amastigotes we had a quite different scenario, despite the high ROS levels observed after clomi treatment, intracellular parasites did not suffer lipoperoxidation (data not shown) neither any loss of cell membrane integrity (Fig. 7E). Although, they still presented increased accumulation of lipid bodies at 30 µM (Fig. 7F).

Clomipramine disrupt the mitochondrial membrane potential (ΔΨ) of *L. amazonensis*

The incubation with clomi tamper with the mitochondrial physiology of *L. amazonensis*. In promastigotes, the uncoupler CCCP reduced the ΔΨ in 35.3% when compared to untreated control, similarly, clomi induced strong dissipation of this parameter, inhibiting in 32.3 and 49.2%, respectively with 8 and 22 µM treatment (Fig. 6A). Despite the alterations observed on mitochondrial physiology, the cellular ATP levels were not affected in promastigotes, even at the higher concentration tested (Fig. 6B). Intriguingly, in amastigotes we have observed analogous effect on the disruption of the ΔΨ (Fig. 7A), but also an additional reduction on the intracellular ATP levels when subjected to Clomi IC_{50} and IC_{90} treatment, similarly as observed for the uncoupler CCCP (Fig. 7B).

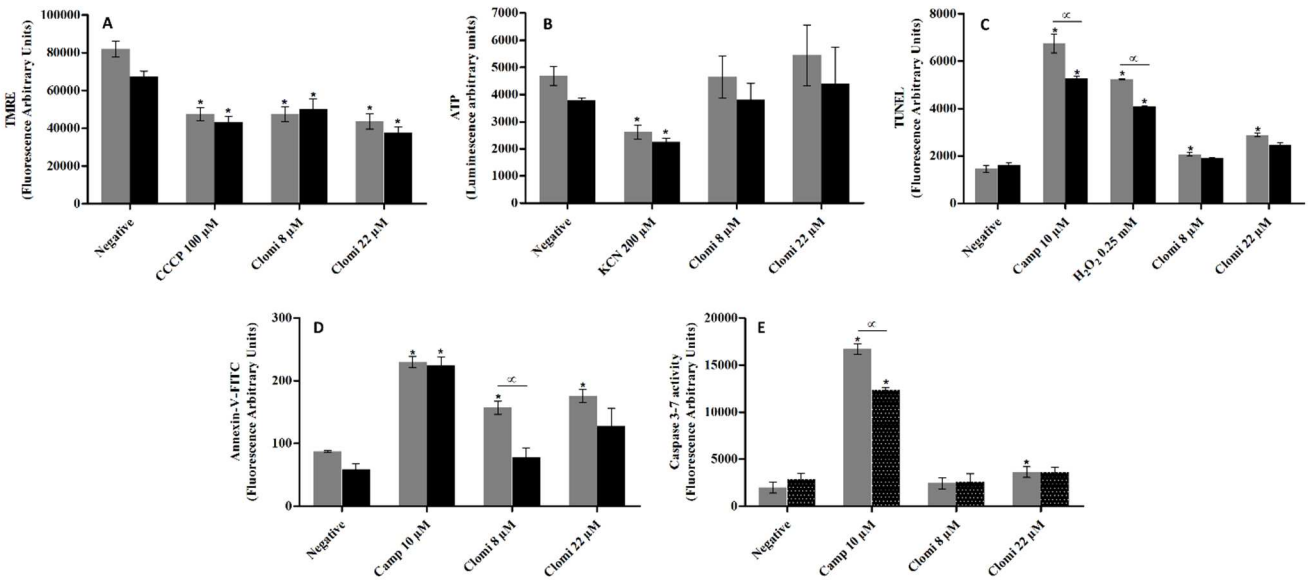

Figure 6. Assessment of mitochondrial metabolism and cell death parameters in promastigotes of *L. amazonensis* treated for 24 h with Clomi (8 and 22 µM). Mitochondrial membrane potential (A) and ATP levels (B) were measured are indicators of energetic metabolism. The DNA fragmentation (C), exposition of phosphatidylserine (D) Caspase3-7 activity (E) were used as apoptosis hallmarks. Gray bars represent parasites treated just with clomi, while black bars (A-D) represent the ones pre-incubated with NAC 200 µM for 3 h before clomi addition, the white dotted black bars represents cell incubated with apotosis inhibitor (Ac-DEVD-CHO) (E). H_2O_2 0.25 mM was used as an oxidative stress inductor, CCCP 100 µM is a mitochondrial uncoupler, KCN 200 µM is a cytochrome c oxidase inhibitor and Camptothecin 10 µM is an apoptosis inductor. * $p \leq 0.05$ compared to untreated control; α represents $p \leq 0.05$ for comparison between the pre-treated and the non-pre-treated with NAC for each treatment.

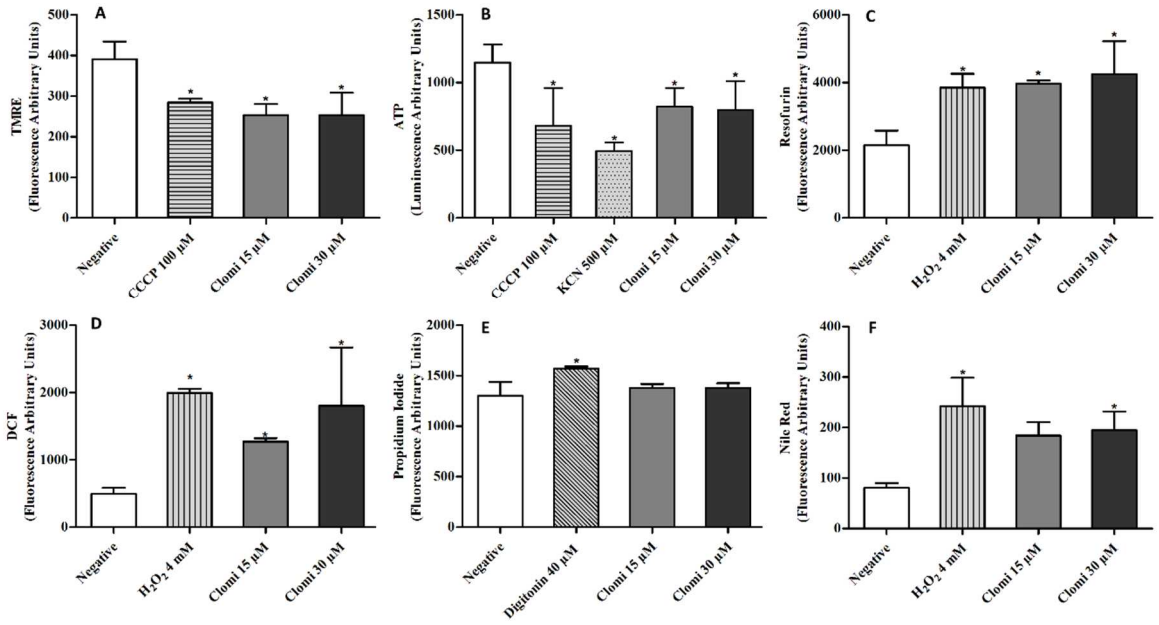

Figure 7. Evaluation of the mechanism of action of clomi (15 and 30 µM) on amastigotes of *L. amazonensis*. Mitochondrial membrane potential (A), ATP levels (B), H_2O_2 production (C), total ROS amounts (D), permeability of plasmatic membrane (E) and lipid droplets accumulation (F) were measured in intracellular amastigotes isolated from macrophages after 24 h treatment. H_2O_2 4 mM is an oxidative stress inductor, CCCP 100 µM is a mitochondrial uncoupler, KCN 500 µM is cytochrome c oxidase inhibitor and Camptothecin 10 µM is an apoptosis inductor. * $p \leq 0.05$ compared to untreated control;

Clomipramine-treated promastigotes exhibit apoptosis hallmarks

The cell death mechanism induced in promastigotes by clomi was also assessed by fluorometric analysis. Promastigotes after 24 h clomi treatment showed strong staining for annexin V-FITC, a PS-exposure marker, increasing in 80.2 and 101.7% the total fluorescence values at 8 µM and 22 µM respectively (Fig. 7D). Another apoptotic attribute evaluated, the caspase 3-7 activity, did not show significant alterations at the lower concentration of Clomi, just when 22 µM was applied that a slight inhibition was observed (Fig. 7E). In addition, our findings indicate that the drug is able to induces DNA fragmentation in a dose dependent way,

with a 42.7% increase at the lower and 98.7% increment at the higher concentration tested (Fig. 7C). The pre-incubation with NAC inhibited in some extent the PS-exposure but did not affect in a significant way the DNA fragmentation induced by Clomi.

Clomipramine induces autophagy on the parasites

In addition, we also performed the MDC staining procedure in order to determine whether autophagy is somewhat related to clomipramine mechanism of action. Treated promastigotes increase in 20.9% and 53.9% the MDC-related fluorescence, for IC_{50} and IC_{90} treated parasites respectively, as an indicative of autophagic vacuole accumulation. As expected, old cultures of promastigotes shown strong autophagy, expressing more than double the MDC-fluorescence when compared to untreated controls (Fig. 8A). For intracellular amastigotes we observed autophagy induction as well, Clomi IC_{50} increased in 55.8 % the fluorescence, while IC_{90} augmented in 64.8 % the autophagic vacuole formation (Fig. 8B).

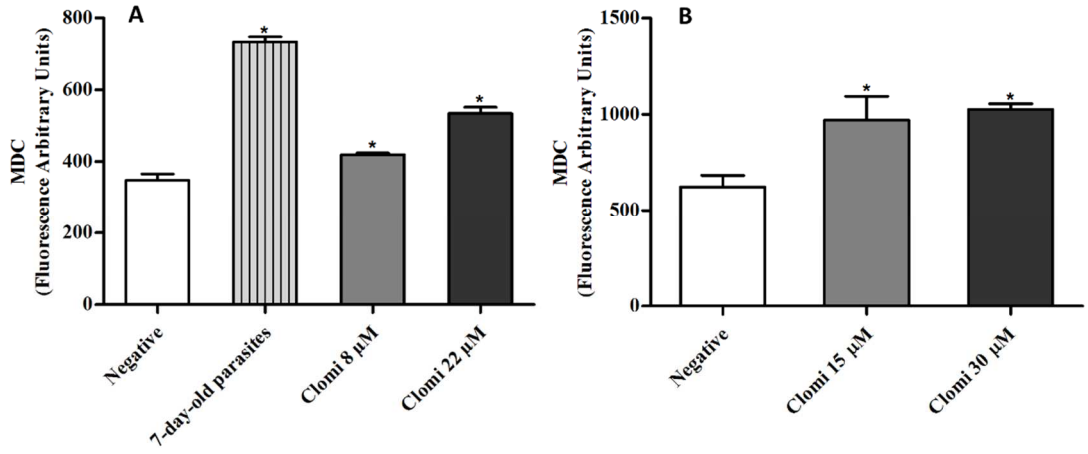

Figure 8. Detection of autophagy in promastigotes (A) and intracellular amastigotes of *L. amazonensis* treated for 24 h with clomi (8 and 22 µM). Monodansycadaverine (MDC) is a marker that accumulates into autophagic vacuoles. 7-day-old parasites were used as positive control for autophagy occurrence. * $p \leq 0.05$ compared to untreated control.

DISCUSSION

Repurposing of drugs is already a successful approach on the discovery of new treatments for several diseases, there are numerous cases of drugs originally conceived for a purpose but later redirected for another. Two of the main drugs currently used on the treatment of leishmaniasis, miltefosine and amphotericin B, were originally developed as antineoplastic and antifungal agents, respectively [14]. On the present work, we proposed the antidepressant clomipramine as a potential drug candidate for repurposing against leishmaniasis. Such research was motivated by previous data, which indicated clomi as a potent inhibitor of tripanosomatids [7,10,11]. Our findings corroborated the expectations, and showed the inhibitory activity of clomi at micromolar concentrations against both, the extracellular and the intracellular forms of *L. amazonensis.* The safety of the drug is assured by the great selective indexes against both the parasitic forms when the activity is compared to toxicity against mammal kidney cells, and mainly by the long time that it is already used on the therapeutic of psychiatric disorders.

The elimination of an intracellular parasite by an antimicrobial agent occurs mainly by two fronts: the direct killing of the parasite by the compound or the stimulation of the host cell in order to combat the microbial intruder. By analyzing the host aspect, we denoted a remarkable stimulation of NO production in macrophages incubated with clomi, together with the antiproliferative activity of the compound such response could effectively contribute to the complete elimination of *L. amazonensis* amastigotes, since this reactive specie has a primordial role on the intracellular parasites eradication [15].

Trypanosomes are microorganisms with peculiar ultrastructure and unique features that differentiate them from host cells, among these characteristics the single and ramified mitochondrion arises as one of the most attractive cellular target for drug development [16]. In order to assess if this organelle could be a target for clomi activity on *L. amazonensis*, we

performed the ultrastructural and biochemical analysis of treated parasites. Our findings indicate that both parasitic forms have their mitochondria affected by clomi, expressing intense mitochondrial membrane depolarization. Interestingly the pre-treatment with the antioxidant NAC did not affect the response induced by the drug on promastigotes, clearly showing that mitochondrial depolarization is a primordial mechanism of action of Clomi, and not a consequence of the oxidative stress that parasites end up. Additionally, TEM analysis also revealed related mitochondrial swelling in treated parasites, alteration that probably appears as a physiological consequence of the ionic imbalance created by the depolarization [17]. Comparable response has been previously observed by treating *Trypanosoma brucei* with clomi [10].

Imipramine, another tryciclic antidepressant, was found to be even more active than miltefosine in vivo against *Leishmania donovani* [18]. Regarding the mechanism of action, the same authors have pointed that imipramine is able to strongly reduces mitochondrial membrane potential of *L. donovani*, similarly to what we have observed in *L. amazonensis*, both in amastigotes and promastigotes. Additionally Higgins and collaborators [19] by studying the effect of tryciclic antidepressants on Glioma cells have also indicated clomi as a strong inhibitor of the mitochondrial membrane potential, and consequently a potent apoptosis inductor, corroborating our hypothesis of mitochondria as a cellular target for this drug.

Once determined that clomi disrupt mitochondrial membrane potential in *L. amazonensis*, and knowing that $\Delta\Psi$ is coupled with the mitochondrial synthesis of ATP [20], we further investigated the intracellular amounts of this energetic molecule. As expected, intracellular amastigotes have their ATP levels in a proportional way affected by the treatment, although, in promastigotes, despite the severe disruption of $\Delta\Psi$, no alteration on the ATP levels has been observed. Such discrepancies, possibly occurs due to metabolic specificities of the two distinct forms of the parasite.

533 It is well established that trypanosomatids pass through different environments and
534 suffer numerous metabolic adaptations during its life cycle [21]. While promastigotes reside in
535 a glucose-rich, slightly alkaline medium inside the insect vector alimentary tract, amastigotes
536 are located mostly into macrophages phagolysosomes, a pretty hostile and acidic milieu where
537 glucose is limited, but great amounts of amino acids and lipids are found [22]. For instance, a
538 proteomic study with *L. donovani* has shown that extracellular promastigotes uses glucose as
539 a main energy source but amastigotes on the other hand, upregulate enzymes involved on
540 amino-acids catabolism, fatty acid β-oxidation and mitochondrial respiration, and uses rather
541 fatty and amino acids as energy matrix [23]. Taken into account the metabolic differences
542 between the parasitic forms, and considering our observations of constant levels of ATP in
543 clomi-treated promastigotes even after $\Delta\Psi$ disruption, it is plausible to believe that substrate
544 level phosphorylation is the mainly responsible for maintenance of ATP levels in these
545 extracellular parasites, similarly as previously described in procyclic *T. brucei* [21].

546 In order to obtain additional information related to mitochondrial physiology of *L.*
547 *amazonensis* treated with clomi we investigate the redox metabolism of these parasites. Both,
548 promastigotes and amastigotes, go into intense oxidative stress after clomi treatment, with
549 initial increased values of total ROS and more specifically huge amounts of hydrogen peroxide.
550 In agreement to previous data [13], our results also suggests that clomi impairs the antioxidant
551 enzyme TR and thus avoids ROS detoxification, contributing to oxidative stress. Further
552 investigations shown that mitochondrial $O_2^{\bullet-}$ just augmented when higher concentrations of
553 drug has been used in promastigotes, giving a clue that formation of nascent superoxide on the
554 electron transport chain is not the main mechanism by which clomi acts initially.

555 We believe that phenomena occurring when the lower dose (i.e. IC_{50}) of clomi has been
556 applied possibly indicates primordial or early steps on its complex mechanism of action. On
557 the other hand, it is clear that if higher concentrations (i.e. IC_{90}) are applied much more

alterations are expected to be observed, possibly due to the widespread cell demise that end up with several secondary effects.

As a consequence of the oxidative stress by which are subjected parasites, promastigotes exhibited high levels of lipoperoxidation already at 8 µM, and even membrane permeabilization and lipid bodies accumulation at the higher dose treatment. The partial inhibition of these effects when the antioxidant NAC was used as pre-treatment, gives some idea in the cause-and-effect relationship between the oxidative stress and the alterations on cell membrane permeability of promastigotes. Intriguingly, the same effect has not been observed in intracellular amastigotes, that although presenting high amounts of ROS even at the lower dose of clomi, did not suffer any lipoperoxidation, neither loss of cell membrane integrity. This particular resilience of amastigotes front of multiple stresses is possibly provided by the slower growth and the stringent metabolism response acquired during evolution as an adaptation to the harsh conditions of the parasitophorous vacuoles [24].

Since multiple alterations have been observed at mitochondrial level, like swelling, ROS generation and mainly membrane depolarization, and considering this organelle as one of the starting sites for apoptotic initiation [25], we investigated the occurrence of programmed cell death (PCD) in clomi-treated promastigotes. Apoptosis, possibly the PCD subtype better described in higher eukaryotes, is characterized by several features, including cell shrinkage, chromatin condensation, chromosomal DNA fragmentation, decrease of mitochondrial membrane potential, exposure of phosphatidylserine residues and activation of caspases [26,27]. Taken together, our results supports the occurrence of apoptosis-like PCD.

Similar occurrence of PCD has been already registered in protozoa parasites [10,16,28]. Nevertheless, even though parasites present morphological features of apoptotic cell death, they lack crucial molecular events like huge caspase activation, the executioners of apoptosis in higher eukaryote [25]. Despite the presence of orthologues of caspases in pathogenic

583 trypanosomatids, called metacaspases, their involvement in cell death is doubtful [29]. This
584 fact might explain the quite low increment of Caspase 3-7-like activity we observed in treated
585 promastigotes, whereas other findings like cell size reduction, membrane shrinkage, DNA
586 fragmentation (TUNEL) and PS exposition clearly indicates occurrence of apoptosis on the
587 parasites even when the lower dose of clomi was applied.

588 In fact, it is suggested that trypanosomatids might have a rather primitive sort of
589 apoptosis that does not primarily depend on caspases but in contrast relies on ROS formation
590 [30]. Our findings corroborate this relationship, since antioxidant pre-treatment successfully
591 prevented the PS exposition in clomi-treated promastigotes.

592 In addition to the mitochondria injury, clomi-treated parasites exhibits also a disturbed
593 endomembrane system, with enlarged endoplasmic reticulum (ER), altered Golgi complex and
594 a flagellar pocket full of vesicles. The relation between apoptosis and the secretory pathway is
595 not completely elucidate, but some evidences indicate that endoplasmic reticulum (ER) and
596 Golgi complex (GC) can act as cell stress sensors and even determine cell fate [31]. Our
597 ultrastructural findings suggests an ER stress scenario after Clomi treatment, what could trigger
598 a pathway named Unfolded Protein Response (UPR) [31]. In occurrence of UPR, these
599 organelles might at first trigger recovery mechanisms by widespread trafficking between cell
600 compartments, activates autophagy components to promote clearance of unwanted or damaged
601 macromolecules and, in case of extensive damage, might even take part on the initiation of
602 apoptosis. As the cell is dismantled during apoptosis, ER vesiculates, and GC disassembles,
603 losing their cisterna arrangement, turning into vesicles and tubular clusters [32,33].

604 The ultrastructural analysis of clomi-treated intracellular amastigotes also revealed the
605 appearance of quite peculiar compartments, characterized as multivesicular vacuoles,
606 surrounded by a single membrane and filled with electron dense cargo. According to the most
607 recent guidelines for monitoring and interpretation of autophagy [34], such observation is close

608 enough to the definition of autolysomes, recognized as autophagy-related structures products
609 of the fusion of double-membrane auto-phagosome with lysosomes. Interestingly, alike
610 structures, have also been previously observed in *T. brucei* subjected to clomi treatment [10].
611 Together with the UPR, autophagy might be understood as a set of adaptive responses that
612 initially aims to recovery an injured cell, but due to extensive damage and compromised cell
613 function terminates by contributing to cell death [26].

614 Additional ultrastructural findings show also that clomi led *L. amazonensis* to intense
615 exocytic activity, what is clearly seen by the numerous vesicles inside the flagellar pocket of
616 treated-parasites. As well-known, the trypanosome flagellar pocket is the region where major
617 exocytosis and endocytosis occurs on the parasite [35] and the accumulation of vesicles could
618 indicate the intense exocytosis of abnormal macromolecules, as denoted when sterol
619 biosynthesis inhibitors are used [36] or the elimination of remnants of autophagic process.

620 As the present research has demonstrated, the antidepressant clomi has great potential
621 as a repurposed agent against *L. amazonensis*. The assured safety of the drug and the activity
622 against both the parasite forms, as well on the activation of macrophages supports further
623 studies on the development of a new therapy for leishmaniasis. Our findings also shed light on
624 the mechanism of action of the drug by indicating that clomi acts through a mitochondrial
625 pathway in *L. amazonensis* (Fig. 9). Both by inhibiting trypanothione reductase activity and by
626 direct interfering on the mitochondrial membrane potential, the drug led parasites to a severe
627 oxidative stress. Autophagy and the remodeling of the endomembrane system possibly appears
628 as an adaptive responses that unsuccessfully attempt to restore cellular homeostasis but
629 culminates with the induction of programed cell death, characteristically apoptosis.
630 Interestingly, differences between the two parasitic forms were observed, with promastigotes
631 in general more susceptible to clomi-induced oxidative stress, and amastigotes, although
632 resilient front of most the effects of ROS accumulation, are rather affected by the mitochondrial

depolarization and consequent decline of ATP level, possibly on accounts of it increased dependence on mitochondrial energetic metabolism. Apart from the specificities, clomi has shown to be able to inhibit effectively *L. amazonensis.*

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COMPETING INTERESTS

All the authors declare no competing interests.

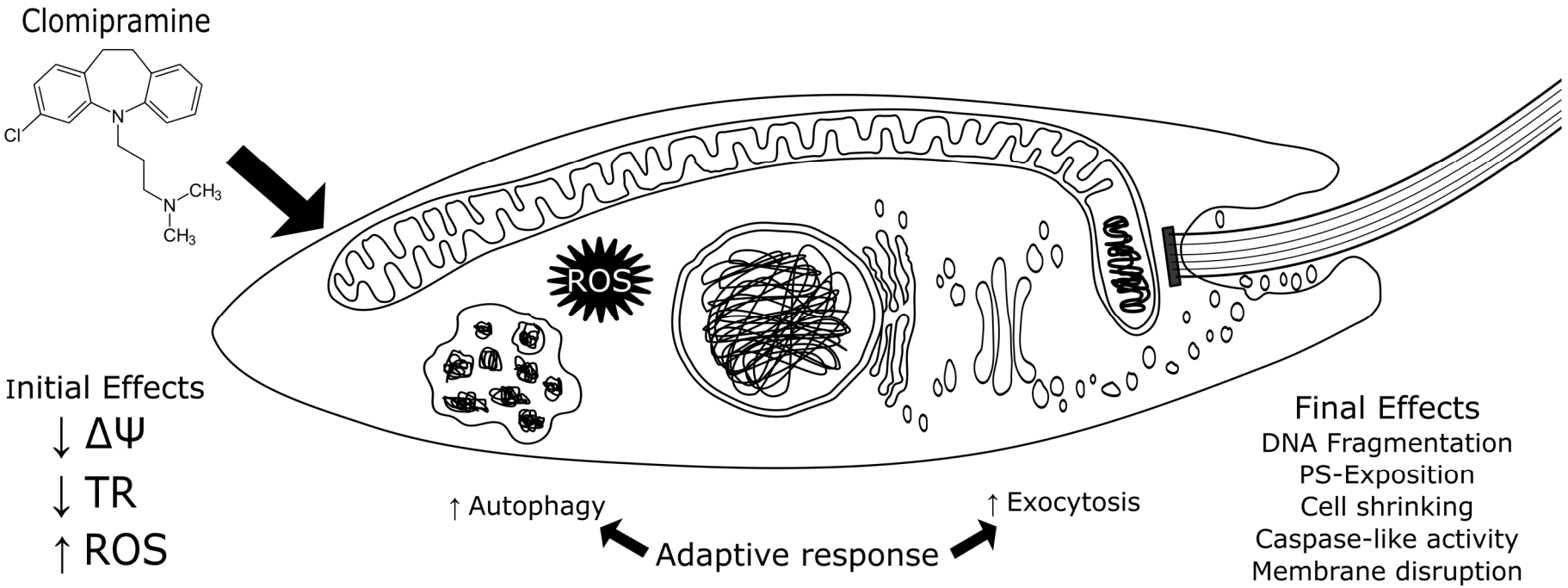

Figure 9. Mechanism of action of clomipramine in *Leishmania amazonensis*. Clomi acts first by inhibiting trypanothione reductase (TR) and impairing the mitochondrial membrane potential (ΔΨ). Parasites are taken to intense accumulation of Reactive Oxygen Species (ROS), and consequently displays autophagy and augmented exocytosis. Parasites end up dying by apoptosis-like programmed cell death, exhibiting several hallmarks.

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