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Clomipramine kills *Trypanosoma brucei* by apoptosis

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ABSTRACT

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

1. Introduction

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

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

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

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

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

2. Methods

2.1. Chemicals

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

2.2. Parasites and cell culture

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

2.3. Anti-proliferative activity

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

2.4. Cytotoxicity assay

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

2.5. Mitochondrial-membrane potential

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

2.6. Reactive oxygen species (ROS)

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

2.7. Phosphatidylserine exposure

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