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# The heme uptake process in *Trypanosoma cruzi* epimastigotes is inhibited by heme analogues and by inhibitors of ABC transporters

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ABSTRACT

Heme (iron protoporphyrin IX) is an important molecule involved in many biological reactions, including oxygen transport, respiration, photosynthesis and drug detoxification. *Trypanosoma cruzi* parasites, the etiological agent of Chagas' disease, take up heme from the environment to supply their nutritional needs because they do not synthesize this cofactor. However, the mechanisms involved in heme transport across biological membranes are poorly understood. Indeed, in *T. cruzi*, no heme transporter has yet been characterized. In the present work, we evaluate the heme uptake processes by *T. cruzi* epimastigotes using fluorescent heme-analogues. Heme uptake decreased significantly when cells were pretreated with different concentrations of SnPPIX, PdMPIX or ZnMPIX, this observed competition suggests that they are taken up by the same transport system. We studied the growth behavior of epimastigotes using the same heme-analogues and the treatments with SnPPIX or PdMPIX impaired cell growth but when heme was added to the culture medium the observed inhibition was partially reversed. In addition, we tested how the heme uptake processes are affected by the presence of different transporter inhibitors. When the cells were treated with inhibitors and then incubated with heme, heme uptake decreased significantly for all treatments. These results constitute a strong indication for the existence of a protein associated with porphyrin transport in *T. cruzi*, possibly ATP-binding cassette transporters (ABC-transporter).


## 1. Introduction

*Trypanosoma cruzi* is a protozoan parasite that resides in the blood and other tissues and causes American Trypanosomiasis, or Chagas' disease, which is recognized by the World Health Organization (WHO) as one of thirteen major neglected diseases throughout the world. Chagas' disease is a significant problem in Latin America (Hotez et al., 2007), where it is endemic, but other countries (considered non-endemic) are considerably affected as well because of the migration movements of the Latin American population (Coura and Viñas, 2010). Currently, an estimated 10 million people are infected worldwide, primarily in Latin America, and more than 25 million people are at risk of being infected (WHO, 2010). The treatment is not very effective; the available medications have serious side effects and little efficacy during the chronic stage of the disease. As there are currently no prospects for vaccines or satisfactory medical treatments, the search for new therapies is a priority. This disease is transmitted by triatomine vector insects while they feed on a vertebrate host. These bloodsucking insects ingest 6–12 times their original weight in blood (Wigglesworth, 1943). This blood is continuously digested through a variety of proteinases, releasing amino acids, peptides and heme. Usually, approximately 10 mM of heme bound to hemoglobin is obtained in a single ingest (Graça-Souza et al., 2006).

The heme molecule is an important cofactor that is involved in several essential biological processes in aerobic organisms, such as oxygen transport (hemoglobin and myoglobin), cellular respiration

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(cytochrome *c*, cytochrome *c* oxidase, and cytochrome *c* reductase), drug detoxification (enzyme P450) and enzymes involved in antioxidant defense (catalases and peroxidases) (Ponka, 1999). Heme is a highly toxic molecule due to its pro-oxidant properties (Ryter and Tyrrel, 2000; Deterding et al., 2004), its biosynthesis and degradation are tightly regulated in accordance with cellular requirements. The heme biosynthetic pathway is conserved throughout evolution, in non-photosynthetic eukaryotic cells, it starts and finishes in the mitochondria, while still involving several cytosolic steps (Severance and Hamza, 2009). There are some organisms that depend on essential heme–proteins but lack in part or in total a heme biosynthetic pathway. Trypanosomatids are included in this group (revisited by Koreny et al., 2010; Tripodi et al., 2011). Biochemical studies have demonstrated the void of a complete heme biosynthesis pathway in *T. cruzi* (Lombardo et al., 2003), and this was later corroborated by the absence of conserved enzymes in its genomic sequence (El-Sayed et al., 2005).As heme is an essential cofactor for trypanosomatids, the processes of heme binding, transport and intracellular distribution must involve critical pathways in these organisms. Heme uptake in trypanosomatids is a topic of actual debate and, specifically for *T. cruzi*, there is limited information concerning transporters or carriers and there is no biochemical characterization of the process itself. Our group has demonstrated that in *T. cruzi* epimastigotes, heme and hemoglobin internalization proceed through different routes and/or mechanisms (Lara et al., 2007). We have observed by fluorescence microscopy using fluorescent heme analogues that heme uptake might involve the activity of a P-glycoprotein (Pgp) homologue, an ABC transporter. Latter, the protein TbHpHbR was described as a receptor for the complex haptohemoglobin-hemoglobin (Hp–Hb) (Vanhollebeke et al., 2008). The authors demonstrated that TbHpHbR is a bloodstream stage-specific protein, but it is not expressed in the procyclic form. TbHpHbR is present in *Trypanosoma brucei subsp. gambiense* and *Trypanosoma brucei subsp. rhodesiense* but absent from the related kinetoplastids *T. cruzi* and *Leishmania*. This protein directs the internalization of heme carried by the Hp–Hb complex into hemoproteins in order to optimize growth of bloodstream forms. Recently, it was demonstrated that an ATP-binding cassette protein was involved in intracellular heme trafficking in *Leishmania* (Campos-Salinas et al., 2011). The protein LABCG5 was associated with the internal distribution of hemoglobin-bound heme to the mitochondria, but LABCG5 was not involved in the internal trafficking of free heme. Currently, the mechanisms by which heme is transported through the plasma membrane and targeted to heme proteins in the intracellular medium remain unknown for all the trypanosomatids.

Considering the implications of new discoveries in the transport of porphyrins in *T. cruzi*, it becomes great importance, especially from the standpoint of chemotherapy, to elucidate the mechanisms for heme transport. In this study, we investigate the effects of heme transport blockage by heme analogues and specific transporters inhibitors and, consequently, the disruption of the life-cycle of *T. cruzi*.

## 2. Materials and methods

### 2.1. Reagents

BHI (brain–heart infusion medium) was obtained from DIFCO (Sparks, MD, USA). Cyclosporin A (CsA) was purchased from Novartis Pharma S.A.S. (Huningue, France). Fetal calf serum (FCS) was purchased from CULTLAB (São Paulo, Brazil). Heme (Fe–protoporphyrin IX), Sn–protoporphyrin IX (SnPPIX), Pd–mesoporphyrin IX (PdMPIX) and Zn–mesoporphyrin IX (ZnMPIX) were obtained from Frontier Scientific (Logan, UT, USA). Indomethacin (1-[p-chlorobenzoyl]-5-methoxy-2-methylindole-3-acetic acid) was obtained from SIGMA–ALDRICH CHEME (Steinheim, Germany). Pyridine was purchased from MERCK (Darmstadt, Germany). Verapamil (5-[N-(3,4-dimethoxyphenylethyl)methylamino]-2-(3,4-dimethoxyphenyl)-2-isopropylvaleronitrile hydrochloride) was also purchased from SIGMA–ALDRICH CHEME (Steinheim, Germany). All other reagents used were of analytical grade.

### 2.2. Parasites

*T. cruzi* epimastigotes, strain Dm28c, were grown at 28 °C for seven days in BHI supplemented with 10% FCS and in the absence or presence of 30 μM heme. The growth was monitored by cell counting in a Neubauer chamber.

### 2.3. Porphyrins

Fe–protoporphyrin IX stock (20 mM) was prepared by dissolving in 0.1 N NaOH, and after it was buffered using PBS (100 mM sodium phosphate buffer and 150 mM NaCl at pH 7.4). The stock was diluted immediately before use to 10 mM in the same PBS buffer. Stocks of Sn–protoporphyrin IX, Pd–mesoporphyrin IX and Zn–mesoporphyrin IX (heme analogues) were prepared in 0.1 N NaOH and buffered in PBS in the same way as the heme and then used in the experiments.

### 2.4. ABC transporter inhibitors (cyclosporin A, indomethacin and verapamil)

The inhibitor stock solutions were prepared as follows: the 5 mM cyclosporin A and 10 mM verapamil stock solutions were prepared in water, and the 10 mM indomethacin stock solution was prepared in 0.1 N NaOH and PBS.

### 2.5. The effect of SnPPIX, PdMPIX, and ZnMPIX on T. cruzi epimastigote heme uptake

The parasites were maintained in BHI supplemented with 10% FCS for 7 days. After 7 days, the cells were washed twice with PBS. After washing, the cells were suspended with PBS and incubated for 10 min with different concentrations of the heme analogues (SnPPIX, PdMPIX, and ZnMPIX). After incubation, they were washed twice with PBS, suspended with PBS and then incubated with 10 μM heme for 10 min. After this incubation, the cells were collected, washed twice with PBS and lysed through repeated freezing and thawing. Heme uptake was measured in epimastigote lysates with the alkaline pyridine method (Falk, 1964) using a GBC UV/VIS 920 absorption spectrometer with the following volume modifications: 500 μL of sample was combined in a 1 mL cuvette with 500 μL of stock solution containing 0.2 N NaOH and 48% pyridine. The oxidized and reduced (via the addition of DTH) spectra were recorded between 500 nm and 600 nm. The 557 and 541 nm peaks were identified in the differential reduced minus oxidized spectra. The heme concentration was estimated using the molar extinction coefficient 20.7 $mM^{-1}$ $cm^{-1}$. As controls we recorded the reduced minus oxidized spectra of heme alone and heme plus different amounts of SnPPIX and we did not observe any difference in the reduced minus oxidized spectra, indicating that these analogues did not show interference in this test.

### 2.6. Analysis of ZnMPIX uptake by T. cruzi epimastigotes measured via direct fluorescence from total cell extracts

The *T. cruzi* epimastigotes were maintained in BHI supplemented with 10% FCS and 30 μM hemin at 28 °C. Parasites in the