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Chemical evaluation of fatty acid desaturases as drug targets in *Trypanosoma cruzi*

Andrés Alloatti [a], Sebastián A. Testero [b], Antonio D. Uttaro [a,*]

[a] *Instituto de Biología Molecular y Celular de Rosario (IBR), CONICET, Departamento de Microbiología, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, 2000 Rosario, Santa Fe, Argentina*

[b] *Instituto de Química Rosario (IQUIR), CONICET, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Santa Fe, Argentina*

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ABSTRACT

Four positional isomers of Thiastearate (TS) and Isoxyl (Thiocarlide) were assayed as fatty acid desaturase inhibitors in *Trypanosoma cruzi* epimastigotes. 9-TS did not exert a significant effect on growth of *T. cruzi*, nor on the fatty acid profile of the parasite cells. One hundred micromolars of 10-TS totally inhibited growth, with an effective concentration for 50% growth inhibition (EC_{50}) of 3.0 ± 0.2 μM. Growth inhibition was reverted by supplementing the culture media with oleate. The fatty acid profile of treated cells revealed that conversion of stearate to oleate and palmitate to palmitoleate were drastically reduced and, as a consequence, the total level of unsaturated fatty acids decreased from 60% to 32%. Isoxyl, a known inhibitor of stearoyl-CoA Δ9 desaturase in mycobacteria, had similar effects on *T. cruzi* growth (EC_{50} 2.0 ± 0.3 μM) and fatty acid content, indicating that Δ9 desaturase was the target of both drugs. 12- and 13-TS were inhibitors of growth with EC_{50} values of 50 ± 2 and 10 ± 3 μM, respectively, but oleate or linoleate were unable to revert the effect. Both drugs increased the percentage of oleate and palmitate in the cell membrane and drastically reduced the content of linoleate from 38% to 16% and 12%, respectively, which is in agreement with a specific inhibition of oleate Δ12 desaturase. The absence of corresponding enzyme activity in mammalian cells and the significant structural differences between trypanosome and mammalian Δ9 desaturases, together with our results, highlight these enzymes as promising targets for selective chemotherapeutic intervention.

1. Introduction

Chagas' disease (American Trypanosomiasis) is caused by *Trypanosoma cruzi*, a hemoflagellate protozoan belonging to the order Kinetoplastida. This parasitic disease represents a serious health problem in the Americas, affecting at least 18 million people with more than 100 million at risk of infection. The only clinically available drugs for the chemotherapy of Chagas' disease are Nifurtimox (Lampit®) and Benznidazole (Rochagan®). Both drugs have important disadvantages such as severe side effects, strain resistance and variable efficacy (Barrett et al., 2003). For these reasons the development of more safe and efficient drugs is urgent.

Trypanosoma cruzi has a life cycle in which the parasite alternates between a reduviid insect vector and the mammalian host. Flagellated epimastigotes proliferate in the insect midgut before differentiating into non-dividing but infectious metacyclic trypomastigotes found in the hindgut. During the bloodmeal on the mammalian host, the infected vector eliminates faeces contaminated with metacyclic trypomastigotes that penetrate the host through skin lesions or mucosa. They promptly invade cells in the vicinity and differentiate into amastigotes, initiating intensive proliferation in the cytosol. Ultimately, the amastigotes develop into non-dividing bloodstream-form (BSF) trypomastigotes that can either initiate another round of infection or be taken up by a reduviid vector during a bloodmeal (Tyler and Engman, 2001). The development of new antichagasic agents may be based not only on rational drug design or on screening of natural products or synthetic compounds but also on taking advantage of compounds already in use against other human diseases, which have passed several of the clinical trials necessary for the development of new drugs.

There is an urgent need to identify specific enzymes and metabolic pathways in the parasite that could be used as potential targets for drug development. These targets have to be present in the mammalian stages of the parasite and in the case for *T. cruzi* which, in the mammal, resides mainly intracellularly, the drugs must be able to enter the host cell to reach the parasite.

Trypanosomatids contain the usual range of lipids also found in their eukaryote host (i.e., triacylglycerols, phospholipids, plasmalogens, sterols) but a higher proportion of polyunsaturated fatty acids (PUFAs) (Mellors and Samad, 1989; Haughan and Goad, 1991). For example, oleate and linoleate can represent up to the 60% of the total fatty acids (FAs) in epimastigote and trypomastigote forms of *T. cruzi* (Florin-Christensen et al., 1997). This suggests a high membrane fluidity that may be essential for the parasites in order to adapt themselves to the dramatic changes in temperature

* Corresponding author. Tel.: +54 341 435 0661; fax: +54 341 439 0465.
E-mail address: toniuttaro@yahoo.com.ar (A.D. Uttaro).

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and chemical parameters experienced during their complex life cycles.

We have recently depicted the pathways for PUFA biosynthesis present in trypanosomatids (Tripodi et al., 2006; Uttaro, 2006; Livore et al., 2007). *Trypanosoma brucei* and *T. cruzi* synthesise oleate (18:1Δ9) and palmitoleate (16:1Δ9) from stearate (18:0) and palmitate (16:0), respectively, by the action of a Δ9 stearoyl-CoA desaturase (SCD) (A. Alloatti and A. D. Uttaro, unpublished data). Oleate is desaturated by the oleate (Δ12) desaturase to linoleate (18:2Δ9,12) (Petrini et al., 2004), which represents the main FA in the cells.

In *Leishmania* species, linoleate can be further desaturated to α-linolenate (18:3Δ9,12,15), and both FAs act as intermediates in the synthesis of 22-carbon PUFAs, by means of three additional desaturases and two elongases. As two of these desaturases and one elongase are absent in *Trypanosoma* species, these parasites synthesise C22 PUFAs by using intermediates taken from the host (Tripodi et al., 2006; Livore et al., 2007). Although these PUFAs represent less than 10% of total FAs, they seem to be important to the cell, as indicated by the fact that part of the pathway has been conserved.

Oleate and linoleate form, together with stearate and palmitate, the bulk of FAs with important structural functions in the cell membrane. We speculate that the endogenous synthesis of these unsaturated FAs is essential for normal growth of the parasite, making SCD and oleate desaturase potential drug targets. In particular, drugs targeted against the latter desaturase may be highly selective, since this enzyme is not present in mammalian cells. Oleate desaturase has been demonstrated to be active throughout the life cycles of *T. cruzi* (Maldonado et al., 2006) and *T. brucei* (A. Alloatti and A. D. Uttaro, unpublished data).

It is relevant to note that glycosylphosphatidylinositol (GPI)-anchored mucins, the major surface antigens of *T. cruzi* BSF trypomastigotes, contain unsaturated (18:1 or 18:2) FAs in their GPI moiety. No unsaturated FAs were found in other GPI-anchored molecules of metacyclic or BSF trypomastigotes or epimastigotes. Mucins and the ceramide-containing glycoinositolphospholipids (GIPLs) of the epimastigote forms contain saturated FAs (Almeida and Gazzinelli, 2001). GPI moieties are involved in the triggering of host innate immunity, which is the initial line of defence against the invading parasite. These glycoconjugates also contribute to the development of acquired immunity by binding to Toll-like receptors (TLRs) on the surface of host macrophages. This initiates a signalling cascade that culminates in the production of pro-inflammatory cytokines. Recent studies have shown that the mucin GPI anchor of *T. cruzi* BSF trypomastigotes activates TLR2 (Campos et al., 2001) whereas GIPLs from epimastigotes activate TLR4 (Oliveira et al., 2004; Gazzinelli and Denkers, 2006). The chain length and degree of saturation of the FA components of GPI are important determinants in the specific binding to TLRs and consequently to their biological activity. There are minor differences between the glycan structures of the mucin GPIs from BSF trypomastigotes and that from epimastigotes. However, BSF trypomastigote GPIs, which contain 18:1 and 18:2 FAs, have potent pro-inflammatory activity whereas the epimastigote and metacyclic trypomastigotes GPIs, which contain saturated (16:0 or 18:0) FAs, are inactive (Almeida and Gazzinelli, 2001; Gazzinelli and Denkers, 2006). This suggests that inhibiting *T. cruzi* FA desaturases could render the parasite more susceptible to the host immune system attack.

There are few known inhibitors of desaturases, and those are not commercially available. Thia fatty acids are FA analogues with sulphur atoms substituting methylene groups in the carbon chain. As introducing sulphur atoms has little effect on the structure of the aliphatic chain, thia fatty acids are metabolized as ordinary FAs and incorporated into different lipid classes (Berge et al., 2002). 9- and 10-isomers of Thiastearates (TS) (Fig. 1A) were shown to be converted to the corresponding acyl-CoAs and to bind to hepatocyte's SCD, causing strong inhibition of Δ9 desaturation (Høvik et al., 1997). The same compounds had been tested earlier on cultures of the trypanosomatids *Crithidia fasciculata* and *Leishmania* spp., although as inhibitors of dihydrosterculic acid biosynthesis (Rahman et al., 1988; Beach et al., 1989). This cyclopropane FA is exclusively synthesised by eukaryotic microbes such as species of *Crithidia, Herpetomonas, Leptomonas* and *Leishmania*, but not by vertebrates, suggesting dihydrosterculic acid biosynthesis as a putative target for selective chemotherapy. 8-, 9-, 10- and 11-TS were all strong inhibitors of dihydrosterculic acid biosynthesis, but with variable effects on the growth of parasites and the FA content of their cell membrane (Rahman et al., 1988; Beach et al., 1989).

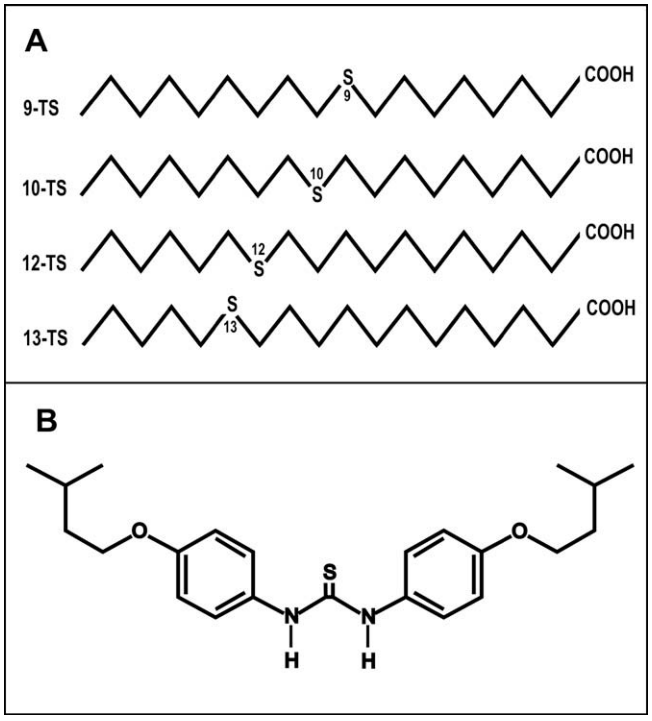

Fig. 1. Structure of drugs used in this work. (A) Thiastearic acid positional isomers (mol. wt. 302). B) Isoxyl (Thiocarlide, mol. wt. 400).

Here, we explore the effect of 9-and 10-TS as putative inhibitors of *T. cruzi* SCD and growth of cultures of epimastigote-form cells of this parasitic species. Indeed, a toxic effect by 10-TS was shown that appears to be related to the inhibition of the Δ9 desaturation, indicating likelihood that this enzyme is essential for normal growth of the parasites. The validation of this target was confirmed by using a chemically unrelated drug, Isoxyl (Thiocarlide) (Fig. 1B), which was recently described as an inhibitor of the SCD of *Mycobacterium tuberculosis* (Phetsuksiri et al., 2003). In addition, we speculated about a specific inhibitory effect of 12- and 13-TS (Fig. 1A) on the Δ12 oleate desaturase. Both isomers were synthesised and indeed showed inhibition of the growth of *T. cruzi* and its oleate desaturase, validating this enzyme as an additional target for selective chemotherapy.

2. Materials and methods

2.1. Materials

Stearate, linoleate, oleate and sodium methoxide were obtained from Sigma (Sigma–Aldrich, St. Louis, MI, USA). All organic solvents