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Lipoic acid metabolism in *Trypanosoma cruzi* as putative target for chemotherapy

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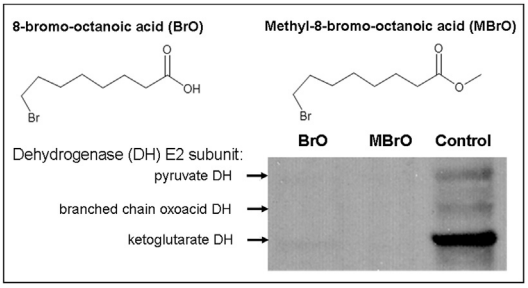
HIGHLIGHTS

- *Trypanosoma cruzi* protein lipoylation is dependent of glucose availability.
- Protein lipoylation is inhibited by lipoic acid analogues.
- Lipoic acid, octanoic acid and 8-bromo-octanoic acid are poorly taken up by *T. cruzi*.
- Methyl-8-bromo-octanoic acid (MBrO) is efficiently incorporated by *T. cruzi*.
- MBrO is an effective growth inhibitor and inhibition is not bypassed by lipoc acid.

GRAPHICAL ABSTRACT



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ABSTRACT

Lipoic acid (LA) is a cofactor of relevant enzymatic complexes including the glycine cleave system and 2-ketoacid dehydrogenases. Intervention on LA *de novo* synthesis or salvage could have pleiotropic deleterious effect in cells, making both pathways attractive for chemotherapy. We show that *Trypanosoma cruzi* was susceptible to treatment with LA analogues. 8-Bromo-octanic acid (BrO) inhibited the growth of epimastigote forms of both Dm28c and CL Brener strains, although only at high (chemotherapeutically irrelevant) concentrations. The methyl ester derivative MBrO, was much more effective, with EC_{50} values one order of magnitude lower (62–66 μM). LA did not bypass the toxic effect of its analogues. Small monocarboxylic acids appear to be poorly internalized by *T. cruzi*: [^{14}C]-octanoic acid was taken up 12 fold less efficiently than [^{14}C]-palmitic acid. Western blot analysis of lipoylated proteins allowed the detection of the E2 subunits of pyruvate dehydrogenase (PDH), branched chain 2-ketoacid dehydrogenase and 2-ketoglutarate dehydrogenase complexes. Growth of parasites in medium with 10 fold lower glucose content, notably increased PDH activity and the level of its lipoylated E2 subunit. Treatment with BrO (1 mM) and MBrO (0.1 mM) completely inhibited E2 lipoylation and all three dehydrogenases activities. These observations indicate the lack of specific transporters for octanoic acid and most probably also for BrO and LA, which is in agreement with the lack of a LA salvage pathway, as previously suggested for *T. brucei*. They also indicate that the LA synthesis/protein lipoylation pathway could be a valid target for drug intervention. Moreover, the free LA available in the host would not interfere with such chemotherapeutic treatments.

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1. Introduction

Several enzyme complexes widely distributed in nature require lipoic acid (6,8-dithiooctanoic acid; LA) as prosthetic group. The most relevant of them are pyruvate dehydrogenase (PDH), 2-ketoglutarate dehydrogenase (KGDH), branched-chain 2-ketoacid dehydrogenase (BCDH) and the glycine cleavage system (GCS) (Perham, 2000; Douce et al., 2001; Spalding and Prigge, 2010). These are involved in important metabolic pathways needed to sustain cell viability. PDH produces acetyl-CoA, driving the pyruvate generated in glycolysis to the tricarboxylic acid (TCA) cycle (Tielens and van Hellemond, 2009) and to the synthesis of fatty acids (Ramakrishnan et al., 2013; Uttaro, 2014), sterols (de Souza and Rodrigues, 2009) and other cell components. Succinyl-CoA produced by KGDH is an intermediate of the TCA cycle, precursor of several amino acids and substrate in the synthesis of porphyrins. Ketoacids derived from the deamination of valine, leucine and isoleucine are decarboxylated by BCDH generating CoA-activated primers used in the synthesis of branched-chain fatty acids (Perham, 2000). GCS catalyses the reversible decarboxylation of glycine with release of ammonia and methylene groups. Methylene groups are transferred to tetrahydrofolate, generating 5,10-methylene-tetrahydrofolate involved in the synthesis of amino acids and nucleotides (Douce et al., 2001). In addition, the decarboxylations described above generate NADH, which can be used in oxidative phosphorylation.

The eukaryotic dehydrogenase complexes are localized in the mitochondrial matrix and are composed of multiple copies of each of three enzymatic subunits referred to as E1 (decarboxylase), E2 (acyl transferase), and E3 (dihydrolipoamide dehydrogenase; DHLDH). Most eukaryotic PDHs contain a fourth subunit (E3Bp), that acts as a linker between E3 and the E2 multimer. LA is covalently attached to the N^6 amino group of a lysyl residue in the lipoyl domain of E2 and to conserved lysyl moieties of E3Bp (Perham, 2000). The subunits of GCS are named P (glycine decarboxylase), H, T, and L proteins. LA is covalently bound to a lysyl moiety of the H protein, which does not have catalytic activity but acts as a scaffold to protect the unstable intermediate during transfer to the T protein, which catalyses the release of ammonia from methyleneamine and the transfer of the methylene group to tetrahydrofolate (Douce et al., 2001). L protein is the DHLDH that regenerates lipoamide; most organisms share the same DHLDH as E3 or L protein in both 2-ketoacid dehydrogenase complexes and GCS.

LA plays an essential role in the catalytic activity of these complexes and any intervention with its synthesis or regeneration would probably lead to a general deleterious effect in the cell. This feature heightens its value as a chemotherapeutic target. As LA metabolism has not been studied in detail in parasitic organisms like trypanosomatids (Spalding and Prigge, 2010), it is our interest to evaluate and validate it as a putative target for drug discovery. Trypanosomatids are flagellated protists belonging to the Kinetoplastida, grouping species like *Trypanosoma brucei* and *Trypanosoma cruzi*, the causative agents of sleeping sickness and Chagas disease, respectively (Barrett et al., 2003). These are considered neglected diseases with elevated morbidity and mortality if not treated. The repertoire of available treatments is limited and most of the drugs used are toxic and, in some cases, ineffective, requiring urgent development of new chemotherapies.

LA metabolism has been described in detail only in some model organisms (Cronan, 2016). *Escherichia coli* for instance, exhibits the simplest route involving the transfer of an octanoyl moiety from octanoyl-acyl carrier protein (octanoyl-ACP) to E2 and H protein by LipB (octanoyl transferase). Subsequently, LipA (lipoate synthase) inserts two sulfur atoms into the octanoyl moiety giving the dithiolane ring of the lipoyl moiety. *E. coli* is also able to scavenge LA; the lipoate ligase LplA uses free LA to acylate E2 and H protein. *Bacillus subtilis* lacks a LipB homologue, but encodes one LipA and three LplA homologues (LplJ, LipM and LipL). One of them, LplJ has the ligase function. This Gram-positive bacterium presents a variation in the synthesis/lipoylation pathway: LipM is an octanoyl transferase that specifically transfers octanoate from ACP to the H protein. LipL is an amidotransferase that transfers the octanoyl moiety from H protein to the E2 subunits of dehydrogenases (Cronan, 2016). *Saccharomyces cerevisiae* encodes Lip2, Lip5 and Lip3, structural homologues of LipB, LipA and LplA respectively. Lipoylation of H protein is required for lipoylation of E2 and it has been suggested that the yeast would be unable to scavenge lipoate, indicating that Lip3 could have amidotransferase activity, like *B. subtilis* LipL (Schonauer et al., 2009). However, it was recently shown that whereas Lip2 is an octanoyl-ACP:protein transferase, apparently specific for H protein, Lip3 is an octanoyl-CoA:protein transferase involved in the acylation of E2 subunits (Hermes and Cronan, 2013).

A survey of trypanosomatid genomes showed the presence of genes encoding subunits of PDH (E1p, E3Bp, E2p), KGDH (E1k, E2k), BCDH (E1b, E2b) and GCS (P, H and T proteins), and a sole DHLDH probably shared by the four complexes. It also revealed the presence of enzymes putatively involved in lipoate synthesis/lipoylation, including orthologs of LipA (or Lip5), LipB (Lip2) and LplA (lip3) (Spalding and Prigge, 2010).

Indirect experimental evidence indicated the absence of lipoate salvage in these protists (Stephens et al., 2007), suggesting that as observed in yeast, LplA or Lip3 homologues could not be true ligases. The lack of salvage however, should facilitate the use of inhibitors of lipoate metabolism in chemotherapy, as the only source of LA for the parasite would be the one produced by the *de novo* pathway. The fact that mammals are highly dependent on LA derived from food and intestinal bacteria makes this feature even more attractive (Bustamante et al., 1998).

It was recently found that the interference of DHLDH expression, which affects the recycling of LA, had a strong proliferation defect in *T. brucei* followed by rapid cell death (Roldan et al., 2011). However, no data about the effect of inhibition on LA synthesis or protein lipoylation are available.

The aim of this work is to validate LA metabolism as a drug target against *T. cruzi*. We show here a chemical approach, by using LA analogues, to study the relative importance of LA biosynthesis and LA salvage in this organism.

2. Materials and methods

2.1. Parasite culture and growth inhibition assays

Epimastigotes of *T. cruzi* CL Brener and Dm28c strains were grown in LIT medium at 28 °C and the culture medium was supplemented with 10% fetal bovine serum. When indicated, modified LIT medium (mLIT) was used, containing 0.4 g/l of glucose instead of the regular amount (4 g/l) (Camargo, 1964). Growth curves were obtained by direct observation and cell counting in a Neubauer haemocytometer, starting from a parasite density of 1×10^6 cells/ml. To study the effect of the LA analogues 8-bromo-octanoic acid (BrO) and methyl-8-bromo-octanoic acid (MBrO) (Fig. 1), parasites were seeded in 24 well plates at 1×10^6 cells/ml and compounds were added at increasing concentrations. Solvent (DMSO) was always at a final concentration of 0.5% (v/v), including the control. Plates were incubated at 28 °C. EC_{50} values were calculated by non-linear regression analysis using SigmaPlot (v 11.0). All experiments were done in triplicate with appropriate controls in each case.

2.2. Synthesis of methyl-8-bromo-octanoic acid

Fifty μg of BrO (Sigma) were dissolved in 2 ml of freshly