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



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#### HIGHLIGHTS

### G R A P H I C A L A B S T R A C T

- *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.

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### ABSTRACT

Lipoic acid (LA) is a cofactor of relevant enzymatic complexes including the glycine cleave system and 2ketoacid 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 EC50 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*: [<sup>14</sup>C]-octanoic acid was taken up 12 fold less efficiently than [<sup>14</sup>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<sup>6</sup> 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 2ketoacid 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 LpIA uses free LA to acylate E2 and H protein. *Bacillus*  subtilis lacks a LipB homologue, but encodes one LipA and three LpIA 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 LpIA 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/lip-oylation, 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, LpIA 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  $1 \times 10^6$  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  $1 \times 10^6$  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<sub>50</sub> values were calculated by nonlinear 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



Fig. 1. Structures of lipoic acid and its analogues.

prepared (2%)  $H_2SO_4$  in anhydrous methanol and incubated during 2 h at 80 °C. The resultant methyl ester derivative (MBrO) was extracted twice with hexane. After drying under a  $N_2$  stream, MBrO was dissolved in DMSO.

#### 2.3. Proteins extraction and Western blots

For the preparation of crude cell lysates, parasites were grown in the corresponding medium and proteins extracted after cell lysis. Briefly, after harvesting, cells were washed once by centrifugation with ice-cold phosphate saline buffer (PBS) and lysed in buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris-HCl, pH 8) in the presence of a cocktail of protease inhibitors (Complete Mini tablets, Roche Applied Science). For mitochondrial enrichment, digitonin membrane permeabilization was done as previously described (Foucher et al., 2006). The protein concentration was determined using BSA as standard. Samples were run in 12% SDS-PAGE gels and blotted onto polyvinylidene difluoride (PVDF) membranes. Proteins were revealed using anti-lipoic acid antibody (Abcam) or anti- $\alpha$ -tubulin antibody (Sigma-Aldrich, St. Louis, USA) and anti-rabbit HRP-conjugated antibodies (Pierce). After washing, blots were processed using the SuperSignal chemiluminescent detection system (Pierce).

#### 2.4. Monocarboxylic acids uptake assay

The protocol was adapted from Voorheis (1980). Briefly, to 500  $\mu$ l of a *T. cruzi* epimastigote culture in LIT medium in its logarithmic growth phase and enriched to a cell density of 2  $\times$  10<sup>8</sup> parasites/ml, 500  $\mu$ l of LIT medium without foetal bovine serum but containing 5  $\mu$ M fatty acid free bovine serum albumin (Sigma), 1  $\mu$ l [1 $^{-14}$ C] octanoic acid (396 290 dpm; New England Nuclear) and 60  $\mu$ M sodium octanoate were added. The cultures were incubated for different periods of time. Incubations were terminated by adding 10 ml PBS at 0 °C and cooled on ice. Cells were harvested, resuspended in 500  $\mu$ l of PBS and transferred to scintillation vials. Incorporated octanoate ( $\mu$ mols) were calculated from total cpms. As a positive control, a similar experiment was carried out by using the same specific activity of [1 $^{-14}$ C] palmitic acid (13 200 dpm/nmole, New England Nuclear). The experiment was done in triplicate.

#### 2.5. Mitochondria isolation and enzyme activity assays

Parasites were grown in LIT and mLIT for 72 h. Additionally, a subset of parasites cultured in mLIT plus  $100 \,\mu$ M MBrO was also included in the study. A modification of the method described by Schonauer et al. (2009) was used. Briefly, for the isolation of a crude

mitochondrial fraction, cells were pelleted by centrifugation at  $3000 \times g$  for 5 min at 4 °C, washed and resuspended in 50 mM Tris-HCl, pH 7.4 in the presence of a cocktail of protease inhibitors. After 3 cycles of freezing-thawing and vortexing, the lysate was homogenized with a 21G syringe and subsequently centrifuged at  $1000 \times g$  for 5 min at 4 °C. The mitochondrial fractions recovered in the supernatants were next lysed by the addition of 0.1% Triton X-100. Protein concentration was determined using BSA as standard. PDH, KGDH and BCDH activities were measured by following the production of NADH at 340 nm at 25 °C and calculations were based on the initial rates. The reaction mixture contained 150 mM Tris/ HCl, pH 7.4, 3 mM cysteine, 0.2 mM CoASH, 4 mM NAD<sup>+</sup>, 1 mM  $MgCl_2$  and 140 µg of mitochondrial extract in a final volume of  $200 \,\mu$ l. The reactions were initiated by the addition of  $10 \,\text{mM}$  of substrate (sodium pyruvate, sodium  $\alpha$ -ketoglutarate or sodium  $\alpha$ ketoisovalerate, respectively). Assays were done in triplicate. Malate dehydrogenase, as a control of cell viability, was assayed as previously described (Hunter et al., 2000).

#### 3. Results and discussion

#### 3.1. LA analogues are inhibitors of T. cruzi growth

We first evaluated the effect of an LA analogue on *T. cruzi* growth and viability. Growth inhibition assays were carried out using T. cruzi epimastigotes of CL Brener and Dm28c strains and parasite viability was assessed by direct microscopic observation and cell counting. Both strains were susceptible to BrO (Fig. 1), showing EC<sub>50</sub>s of 0.470 + 0.010 mM (CL Brener) and 0.885 + 0.015 mM (Dm28c) (Fig. 2). The effect of BrO on cell growth and lipoylation profile has been previously studied in T. gondii (Crawford et al., 2006) and P. falciparum (Allary et al., 2007). The addition of this compound to cultures of these organisms drastically inhibited parasite replication and LA supplementation was able to partially rescue the death phenotype. Although the effective BrO concentration was different for each of these parasites (approximately  $10 \,\mu\text{M}$  and  $100 \,\mu\text{M}$ , respectively), the ability of LA to revert the growth phenotype indicated that apicomplexan parasites have an active LA scavenging route. The mechanism of BrO inhibition was shown to require the action of LipB, which catalyses the ligation of the analogue to the apo-protein and produces an inactive intermediate that cannot be used as substrate for LipA and hence cannot produce the active lipoyl-group.

We also tested whether LA addition to a *T. cruzi* culture would be able to influence the parasite's response to 1 mM of the inhibitor. Interestingly, 1 mM of LA appears to be toxic, with evident growth inhibition after 48 h of treatment. Simultaneous supplementation of LA and BrO to the Dm28c strain showed an additive deleterious effect (Fig. 3A). A similar result was obtained by using the most susceptible CL Brener strain and 0.5 mM of each, LA and BrO (Fig. 3B). Lower concentrations of LA were not toxic, but did not bypass the effect of BrO.

### 3.2. T. cruzi epimastigotes cannot efficiently incorporate small monocarboxylic acids

The relative high  $EC_{50}$ s determined for both *T. cruzi* strains may be an indication that BrO is not effectively incorporated into the cells. Due to the unavailability of radioactive LA or BrO, we used the commercially available [<sup>14</sup>C] octanoate, in order to evaluate the uptake of related small monocarboxylic acids by *T. cruzi* (Fig. 4). We could not detect significant amounts of radioactive label associated to cells, which indicated that free octanoate was not actively transported across the plasma membrane. As a positive control, the uptake of [<sup>14</sup>C] palmitate was also assayed, as this fatty acid was



Fig. 2. Growth curves of *T. cruzi* epimastigotes cultured with increased concentrations of 8-bromo-octanoic acid (BrO). The CL Brener (A) and Dm28c (B) strains were tested. Closed and open circles in the right hand panels represent the values of each duplicate at 48 h incubation.

previously shown to be readily incorporated by trypanosomatids, presumably using specific transporters (Voorheis, 1980; Uttaro, 2014).

# 3.3. MBrO has increased membrane permeability and exhibits higher toxicity to trypanosomes

Our data have shown that T. cruzi cannot avidly incorporate octanoate and, most probably, neither LA and BrO from the external medium, which is in agreement with the lack of a LA scavenging route. This result validates the high  $EC_{50}$  values obtained when cultures were exposed to BrO. This also suggests that LA and LA analogues could only be partially incorporated into the cell as a result of very inefficient passive diffusion. Under these circumstances, the chemotherapeutic relevance of the inhibitor is clearly reduced as the amount of it needed to meaningfully reduce parasite viability is considerably high (millimolar range). Therefore, we modified the BrO molecule to its methyl ester derivative MBrO (Fig. 1) to increase its permeation across the parasite cell membrane. Both parasite strains were significantly more sensitive to treatment with this derivative when compared to BrO (Fig. 5), with EC<sub>50</sub> values more than one order of magnitude lower being  $66.2 \pm 10.4$  and  $62.2 \pm 7.5 \,\mu\text{M}$  for CL Brener and Dm28c strains, respectively. The toxic effect of MBrO on CL Brener strain was not bypassed by LA addition when assayed at  $100 \,\mu\text{M}$  (Fig. 6), a concentration at which it did not show significant deleterious effect when administered alone.

#### 3.4. LA analogues block protein lipoylation

LA metabolism and protein lipoylation were also altered by BrO and MBrO. Fig. 7 shows the lipoylation profile of *T. cruzi* parasites (Dm28c strain) grown in the absence and presence of 1 mM BrO or 100  $\mu$ M MBrO for 72 h. Bands corresponding to the E2 subunits of PDH (E2p), BCDH (E2b) and KGDH (E2k) were revealed in a Western blot with a polyclonal antibody that detects lipoyl moieties. Sublethal concentrations of both compounds were able to fully inhibit protein lipoylation, indicating that BrO and MBrO could inhibit the acyl-transfer step or the introduction of sulfydryl groups catalyzed by a lipoyl synthase.

#### 3.5. T. cruzi lipoylation pattern is regulated by growth conditions

A curious result of the previous section (Fig. 7) was the weak signal for the lipoylated E2 subunits of BCDH (E2b) and PDH (E2p, most notable). Although the insect stages of T. cruzi uses L-proline as the main carbon and energy source, the parasite favors the use of glucose, if it is available, over the amino acid (Bringaud et al., 2012). The sugar is metabolized via the glycolytic pathway that takes place mainly inside glycosomes, leading to the production of succinate that is excreted into the medium (Maugeri et al., 2011). A significant fraction of the glycolytic flux, however, goes to the synthesis of pyruvate by the action of cytosolic pyruvate kinase; pyruvate is transported into the mitochondria and converted into acetyl-CoA by PDH. In T. brucei, acetyl-CoA is used for substrate level phosphorylation, producing ATP by two coupled reactions catalyzed by the acetyl:succinate CoA transferase and succinyl CoA synthetase. Genes encoding both enzymes are also present in the T. cruzi genome. When glucose is not present, proline can be metabolized to 2-ketoglutarate and subsequently converted into succinyl-CoA by the action of KGDH (Bringaud et al., 2012). It can be concluded that both PDH and KGDH are relevant enzymatic complexes to support growth of T. cruzi epimastigotes in standard culture media like LIT (rich in glucose and aminoacids) and therefore a robust expression of subunit components of both dehydrogenases is expected. We decided to evaluate the effect of glucose content on



**Fig. 3.** Effect of lipoic acid on cultures of *T. cruzi.* (**A**) Epimastigotes of *T. cruzi* Dm28c strain were grown in the absence or presence of 1 mM BrO, in LIT medium supplemented with lipoic acid (LA, 1 mM). (**B**) Epimastigotes of CL Brener strain grown in the absence or presence of 0.5 mM BrO and/or 0.5 mM LA.

parasite protein lipoylation. Cells were cultivated in media containing regular (4 g/l) or low (0.4 g/l) glucose concentration (LIT and mLIT media, respectively) and growth rates were followed over time. In mLIT medium parasite growth reached stationary phase faster (96 h) than parasites grown in LIT (Fig. 8A). Culture lysates taken at 72 h (logarithmic phase) were run on SDS-PAGE and analyzed by Western blot to detect lipoylated proteins. Notably, parasites grown at low glucose concentration presented increased E2p lipoylation when compared with control cultures (Fig. 8B, lanes mLIT and LIT, respectively). These data indicate that at least T. cruzi E2p expression and/or lipoylation can be modified by growth conditions. Additionally, protein lipoylation was still significantly inhibited when cells cultivated in mLIT were treated with  $100 \,\mu M$ MBrO (Fig. 8B, lane mLIT + MBrO). Mitochondria-enriched fractions were obtained using digitonin, a detergent that based on cholesterol content allows progressive membrane permeabilization. Mitochondria-enriched fractions were analyzed for the presence of lipoylated proteins (Fig. 8C). Similar to our previous observations, lipoylated E2p was increased in T. cruzi grown in mLIT and a



**Fig. 4.** Monocarboxylic acid incorporation into *T. cruzi* epimastigote cells. Cells from Dm28c strain were incubated at 30 °C during different periods with 30  $\mu$ M [1–<sup>14</sup>C] octanoic acid or 30  $\mu$ M [1–<sup>14</sup>C] palmitic acid, both at the same specific activity (13200 dpm/nmole).



**Fig. 5.** Growth curves of *T. cruzi* epimastigotes cultured with increased concentrations of methyl-8-bromo-octanoic acid (MBrO). The CL Brener (**A**) and Dm28c (**B**) strains were tested.



**Fig. 6.** Effect of lipoic acid on cultures of *T. cruzi* grown in the presence of methyl-8bromo-octanoic acid (MBrO). Epimastigotes of *T. cruzi* CL Brener strain were grown in the absence or presence of 66  $\mu$ M MBrO, in LIT medium supplemented or not with lipoic acid (LA, 100  $\mu$ M).



**Fig. 7.** Western blot analysis of lipoylated proteins in *T. cruzi* epimastigotes (Dm28c strain) grown in LIT medium with or without LA analogues. BrO, 8-bromo-octanoic acid; MBrO, methyl-8-bromo-octanoic acid; E2p, E2b and E2k indicate the lipoylated E2 subunits of PDH, BCDH and KGDH complexes, respectively (their theoretical molecular masses are indicated).

substantial decrease in protein lipoylation was seen after supplementation with sublethal MBrO concentration.

The effect of BrO on *T. cruzi* grown in low glucose media was also tested. This was achieved by challenging cultures with increasing concentrations of the compound (Fig. 9). Two types of parasite populations were tested: the first one derived from epimastigotes grown in LIT medium, which were washed and cultivated in mLIT (LIT/mLIT), and a second one obtained by successive passages of cell populations grown in mLIT (71 passages, during ca. 9 months). In both cases similar  $EC_{50}$ s were obtained:  $1.26 \pm 0.04$  and  $1.33 \pm 0.11$  mM, respectively. This represents an increase of 1.5-1.6 fold compared to the  $EC_{50}$ s shown in section 3.1, suggesting an increased amount of the compound's target once cells were adapted to growth in low glucose medium.

## 3.6. Oxoacid dehydrogenase enzyme activities were completely inhibited by MBrO

*T. cruzi* parasites grown in LIT, mLIT and mLIT plus the inhibitor MBrO were assayed for the three LA-dependent oxoacid



**Fig. 8.** Effects of low glucose availability on growth (**A**) and protein lipoylation (**B**, **C**) of *T. cruzi* epimastigotes. LIT, normal LIT medium (4*g*/l glucose); mLIT, modified LIT medium (0.4 *g*/l glucose). (**A**) Growth curves of *T. cruzi* epimastigotes in LIT and mLIT medium. (**B**) Western blot of cells grown in mLIT in the absence or presence of MBrO, compared to cells grown in LIT.  $\alpha$ Tub:  $\alpha$ -tubulin, as loading control. (**C**) Mitochondrial proteins enrichment after digitonine titration; the last two fractions for each treatment are shown.

dehydrogenase activities (Table 1). At low glucose concentration, PDH activity was 5 times higher than the one measured in regular LIT medium. These data nicely correlate with the lipoylation pattern detected under the same growth conditions (Fig. 8). In accordance with the Western blot results, the KGDH specific activity was unaltered by the medium composition while BCDH exhibited very low values under all tested conditions, indicating that both their activities and lipoylation patterns are independent of the glucose concentration. MBrO abolished completely the three enzyme activities, no matter the medium composition. Malate dehydrogenase specific activity was not significantly altered by any of the treatments described above (data not shown), indicating that only lipoylated enzymes were affected.

#### 3.7. Conclusions

Protein lipoylation in trypanosomes has been poorly studied to date, although the great number of metabolic pathways involved and/or associated to LA metabolism underscores its relevance in cell biology and highlights it as a promising chemotherapeutic target. Results compiled in this work support the hypothesis that LA metabolism is essential for *T. cruzi* survival and validates the



Fig. 9. Growth curves of T. cruzi epimastigotes (Dm28c strain) cultured with increased concentrations of BrO. LIT/mLIT indicates cells that were grown in LIT medium before being washed and inoculated into mLIT medium; mLIT indicates populations of cells obtained after repeated (71) passages in mLIT medium.

#### Table 1

Enzymatic activities of mitochondrial dehydrogenase complexes of T. cruzi epimastigotes.

Substrate	Growth condition		
	LIT medium	mLIT medium	mLIT medium, MBrO
pyruvate 2-keto-glutarate 2-keto- <i>iso</i> valerate	$\begin{array}{c} 0.61 \pm 0.11^{a} \\ 0.58 \pm 0.08 \\ 0.15 \pm 0.05 \end{array}$	$\begin{array}{c} 3.01 \pm 0.23 \\ 0.60 \pm 0.10 \\ 0.21 \pm 0.06 \end{array}$	ND <sup>b</sup> ND ND

mU, nmoles/min/mg of protein. b

Not detected, less than 0.1 mU.

lipoylation pathway as a new drug target. Our data also suggest that the parasite cannot efficiently incorporate LA and LA analogues. Although this is in agreement with previous observations that T. brucei lacks LA salvage (Stephens et al., 2007), it is clear that additional experiments using labelled LA are needed in order to confirm the absence of LA uptake and LA transfer to dehydrogenase subunits. It is also important to note that host LA would not interfere with a chemotherapy based on LA analogues. This is because mammals are highly dependent on LA provided by food and enterobacteria (Bustamante et al., 1998), which should make a LA analogue based therapy less toxic for the host.

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