Available online at www.sciencedirect.com
SCIENCE DIRECT
Experimental Parasitology xxx (2006) xxx–xxx
Experimental Parasitology
www.elsevier.com/locate/yexpr

Leishmania amazonensis: Metabolic adaptations induced by resistance to an ABC transporter blocker

Claudia Machuca, Adriana Rodríguez, Marymar Herrera, Sonia Silva, Alicia Ponte-Sucre *

Laboratory of Molecular Physiology, Instituto de Medicina Experimental, Facultad de Medicina, Universidad Central de Venezuela, Caracas, Venezuela

Received 28 November 2005; received in revised form 30 January 2006; accepted 6 February 2006

Abstract

We compared growth rate, cell glucose turnover and expression of *A*TP-*b*inding-*c*assette (ABC) transporters in *Leishmania amazonensis* (LTB0016; LTB) versus LTB^{160} selected for resistance against the ABC transporter blocker glibenclamide. Additionally, we evaluated the influence of drug-resistance on *Leishmania* sensitivity against 2-mercaptoacetate and 2-deoxyglucose. Our data demonstrate that (1) LTB^{160} and LTB constitutively express ABC transporters for neutral substrates, (2) glibenclamide resistance induces the expression of organic anion ABC transporters, members of the drug resistance associated transporters subfamily, (3) LTB^{160} parasites use less glucose as energy substrate and exhibit a slower glucose uptake than LTB cells, and (4) LTB^{160} parasites are less sensitive to 2-mercaptoacetate and 2-deoxyglucose than the glibenclamide-sensitive *Leishmania* LTB. Together these and previous results indicate that the metabolic adaptations expressed in drug-resistant LTB^{160} differ from those described for mammalian drug resistant cells and constitute general mechanisms that underlie drug resistance in *Leishmania* and may be helpful for identifying alternative strategies to circumvent drug resistance in leishmaniasis.

Index Descriptors and Abbreviations: P-glycoprotein; Multidrug associated protein; *Leishmania* (*L.*) *amazonensis*; Calcein-acetoxymethyl ester; Glucose accumulation

1. Introduction

The therapeutic approach against leishmaniasis is mainly carried out with pentavalent-antimoniate-(SbV)-containing agents like Glucantime and Meglumine, despite their toxic side effects, therapeutic failure, and increasing drug resistance. Alternative therapies are unfortunately limited. Restricted knowledge of the mechanisms that underlie drug resistance in *Leishmania* constitutes a major obstacle for the design of alternative therapies against leishmaniasis (Ponte-Sucre, 2003). In vitro work suggests that *A*TP-*b*inding-*c*assette (ABC) transporters are involved in drug resistance in *Leishmania* (Jones and George, 2005; Légaré et al., 2001), but the associated metabolic changes are poorly understood.

Glibenclamide (GLIB) is a sulfonylurea that inhibits ABC proteins with dissimilar functions, such as the K^+-ATP channel associated sulfonylurea receptor (Inagaki et al., 1995), the cystic fibrosis transport regulator (Schultz et al., 1996), the ABC1 transporter of immune cells (Becq et al., 1997; Hamon et al., 1997), the P-glycoprotein (P-gp) (Golstein et al., 1999), the *Arabidopsis* multidrug resistance-related protein AtMRP5 (Lee et al., 2004), and the multidrug resistance associated protein (MRP) (ABCC1) of cancer cells (Conseil et al., 2005).

In vitro selection of *Leishmania* (*Sauroleishmania*) *tarentolae* for resistance to metals (SbV and arsenite) allowed the description of *LtpgpA* and a subsequent group of genes belonging to the same family (Borst and Ouellette, 1995; Légaré et al., 1994; Ouellette et al., 1990). The *LtpgpA*

* Corresponding author. Present address: Institut für Molekulare Infektionsbiologie. Universität Würzburg, Röntgenring 11, D-97070 Würzburg, Germany. Fax: +49 931 312578.
E-mail addresses: aiponte@reacciun.ve, alicia.ponte-sucre@mail.uni-wuerzburg.de (A. Ponte-Sucre).

0014-4894/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.exppara.2006.02.008

protein product PGPA is the most divergent eukaryotic ABC transporter protein described up to now (Ouellette et al., 1990); furthermore, PGPA exhibits close relation to MRP (Cole et al., 1992) and is included in the subset of MRP transporters sensitive to GLIB (Conseil et al., 2005; Payen et al., 2001). Besides the *Ltpgp* family, genes homologous to the human gene-encoded multidrug resistance P-gp (Gottesman et al., 1995; Higgins, 1992; Juliano and Ling, 1976) have also been described in diverse *Leishmania* species (Chow et al., 1993; Henderson et al., 1992; Katakura et al., 1999).

Glucose is the major source of energy for *Leishmania* sp., but although small proportions of carbohydrates are oxidized completely to carbon dioxide via the Krebs cycle, glycolysis is always active (Tielens and Van Hellemond, 1998). However, *Leishmania* sp. has a poor capacity for anaerobic function and depends mainly on respiration for energy generation (Tielens and Van Hellemond, 1998).

A Venezuelan *Leishmania* selected for resistance to GLIB [NR(Gr)] grows similarly to wild type [NR(Gs)] cells; however it exhibits less use of glucose as energy substrate, less glucose uptake and less glucose transporter expression than the GLIB-sensitive parasites at their exponential phase of growth (Uzcategui et al., 2005). Additionally, the activity of enzymes involved in the initial and central part of glucose metabolism, such as hexoquinase (HK) and, especially, phosphoglucose isomerase (PGI) and pyruvate kinase (PK), decreases in a coordinated way without alterations in the activity of glyceraldehyde-3-phosphate dehydrogenase (García et al., 2000; Uzcategui et al., 2005). HK and PGI (intra-glycosome), as well as PK (cytosol), belong to a group of enzymes with specific activities that change in a coordinated way when growth rates change in *Leishmania donovani* (ter Kuile, 1999). Finally, drug-resistant *Leishmania mexicana* produces similar levels of ATP (Singh and Lee, 1999) as wild type cells, suggesting that drug resistance in *Leishmania* does not primarily modulate respiration.

These results suggest that the metabolic adaptations of drug-resistant *Leishmania* differ from those described for mammalian resistant cells, i.e., increased glucose transport and metabolism and increased oxygen consumption (Harper et al., 2002; Lyon et al., 1988; Martell et al., 1997; Vera et al., 1991).

Herein we compared growth rate, cell glucose turnover, and expression of ABC transporters in a reference *Leishmania amazonensis* strain (LTB0016; LTB) versus its counterpart selected for resistance against GLIB (LTB^{160}). Additionally we evaluated the influence of drug resistance on the sensitivity of *L. amazonensis* against the antimetabolites 2-mercaptoacetate and 2-deoxyglucose.

Our data demonstrate that metabolic adaptations that underlie drug resistance in *Leishmania* differ from those described for mammalian drug-resistant cells and may constitute general mechanisms that underlie drug resistance in this parasite. The understanding of this mechanism may be helpful to identify strategies to circumvent *Leishmania* drug resistance and design therapies for the successful treatment of leishmaniasis.

2. Materials and methods

2.1. Reagents

Drugs and chemicals were purchased from the following companies: the fluorescent substrate 2-deoxyglucose (*2-DOG) and the Vybrant™ multidrug resistance assay kit (V-13180), from Molecular Probes, Inc. (Eugene, OR); GLIB, from Aldrich International Co. (St. Louis, MO); the 170-UV kit, from Sigma Chemical Co. (St. Louis, MO); the additional analytical grade chemicals, from Sigma Chemical Co. and Gibco Life Technologies, (Carlsbad, CA).

The kit V-13180 included the following reagents: Calcein acetoxymethyl ester (CAL-AM) (at least 91% pure), verapamil, and cyclosporine-A. CAL-AM was dissolved in dimethyl sulfoxide (DMSO) (1 mM stock solution), and stored at −20 °C; verapamil, and cyclosporine-A were dissolved in pure ethanol (20 mM and 8 mM stock solutions, respectively), and used immediately. GLIB was prepared in DMSO (100 mM stock solution) and stored at 4 °C.

2.2. Cell culture

Leishmania (*L.*) *amazonensis* LTB0016 (MHOM/BR/77) (LTB) is a WHO reference strain. It was kindly provided by Dr. Lee Schnurr, University of Jerusalem, Israel. We cultured *Leishmania* promastigotes at 26 °C, in blood agar semisolid media-RPMI 1640, supplemented with 2×10^{-5} M L-glutamine, 20 μg ml^{-1} gentamicin, 2×10^{-5} M β-mercaptoethanol, and 10% fetal bovine serum. The resistant strain LTB^{160} was selected in vitro by successive passage of the parental sensitive strain LTB (Ponte-Sucre et al., 1998), according to protocols designed previously to induce resistance in the Venezuelan *Leishmania* strain NR (MHOM/VE/80/NR) (Ponte-Sucre et al., 1997). The selection of the resistant strain was performed in the presence of $16–25 \times 10^{-6}$ M GLIB (free drug concentration) as described (Ponte-Sucre et al., 1997). LTB^{160} was further maintained under the pressure of 25×10^{-6} M GLIB; the experiments were performed in the absence of GLIB unless otherwise indicated.

2.3. Kinetics of 2-deoxyglucose accumulation

To determine glucose accumulation we used a previously described protocol (Burchmore and Hart, 1995) with minor modifications. Cells were washed twice and resuspended in 20 mM Hepes, 132 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, and 0.5 mM $MgCl_2$, pH 7.3 (Hepes buffer) at a cellular density of 2×10^8 cells ml^{-1}. We used the non-metabolizable, fluorescent-labeled glucose analogue *2-DOG, that is transported by *Leishmania*. To avoid *2-DOG intracellular phosphorylation (Schaeffer et al., 1974), hydrolysis (ter Kuile and Opperdoes, 1993) or sequestration by the

glycosome (Burchmore and Hart, 1995), aliquots (100 µl) of cell suspension were incubated with 10 mM sodium azide in a shaking water bath, 30 min at room temperature (RT), 5 min at 30 °C. Assays were initiated by the addition of 100 µL of 12 µM fluorescent 2-deoxyglucose (*2-DOG) and ended at various time points by the addition of Hepes buffer-1% formaldehyde and high speed centrifugation at 4 °C, 13,000*g*, 20 s. The supernatant was aspirated, and the pellet was washed with 1 ml of ice cold Hepes buffer and centrifuged again; then we solubilized the pellet in 200 µL of 0.2 M NaOH in 0.1% Triton X-100 and monitored the fluorescence signal in a Perkin Elmer Victor II spectrofluorimeter at 540 nm ($\lambda_{ex} = 465$ nm).

2.4. Glucose, ammonium, and protein determination

We determined D-glucose concentration by the hexokinase/ glucose 6-phosphate dehydrogenase method (Seyfang and Duszenko, 1991). The ammonium concentration was determined with the 170-UV kit. Protein concentration was determined by the dye-binding method (Bradford, 1976), using serum albumin as standard. All the determinations were made in triplicate for each experiment.

2.5. Inhibition of Leishmania growth by 2-mercaptoacetate and 2-deoxyglucose

Cells were seeded at 2×10^6 ml^{-1} alone or in the presence of 2-mercaptoacetate (2-MA) or unlabeled 2-deoxyglucose (UL-2-DOG). Parasite growth was then followed daily. For both cell lines the exponential phase was taken from day 1 to day 4 and the stationary phase from day 5 to day 7. Changes in medium concentration of glucose or ammonium were calculated as the difference in glucose concentration between the beginning and the end of the exponential phase of growth, corrected by the number of cells present in the culture and expressed as mmoles per 10^9 cells. The supernatant was collected and stored at −20 °C until glucose and ammonium concentrations were determined. Glucose concentration in fresh medium was 12.620 ± 0.154 mM.

2.6. Kinetics of calcein extrusion

GLIB-resistance in the Venezuelan strain *L. amazonensis* NR(Gs) is associated with the amplification of DNA sequences related to *ltpgpA* (Ponte-Sucre et al., 1997), as well as to the increased expression of proteins recognized by antibodies raised against the N-terminal of MRP (García et al., 2000). To evaluate the function of ABC proteins related to drug resistance in *Leishmania* LTB and LTB^{160} we measured the rate of CAL-AM and calcein (CAL) transport in LTB and LTB^{160}. CAL-AM is the hydrophobic non-fluorescent precursor of CAL; it freely diffuses into cells, is hydrolysed intracellularly by non-specific esterases, and the resultant negative charged metabolite is trapped within the cells. ABC transporters associated with drug resistance extrude CAL-AM or CAL from the cell. To measure CAL-AM and CAL transport we used the protocol (Essodaïgui et al., 1998, 1999) with minor modifications. Cells were washed twice and resuspended in Hepes buffer at a cellular density of 1×10^8 cells ml^{-1}. Aliquots (100 µl) of cell suspension were incubated with 5 mM glucose (energized) or 10 mM sodium azide (ATP depleted) for 30 min at RT. The fluorescence signal from cells incubated at different concentrations of CAL-AM was monitored continuously for 100 min on a Perkin Elmer Victor II spectrofluorimeter at 517 nm ($\lambda_{ex} = 494$ nm) in. Cells were further incubated in the presence of 2 µM cobalt (Co^{2+}), which quenches the extracellular CAL; the fluorescence signal was further monitored for 30 min. The permeability and toxicity of different concentrations of Co^{2+} towards promastigotes was determined in parallel experiments; as has been demonstrated previously, 2 µM Co^{2+} does not permeate and is not toxic to the parasites (Essodaïgui et al., 1998). To quantify the self quenching of intracellular CAL we incubated the cells in 2.3 µM CAL-AM and monitored the fluorescence until a plateau was reached; afterwards we permeabilized the cells with 0.01% Triton X-100 and measured the change in fluorescence for additional 30 min. The appropriate fluorescence signal corrections were performed for each strain.

2.7. Inhibition of calcein cell-retention

To measure calcein cell-retention we used the protocol supplied with the Vybrant™ multidrug resistance assay kit with minor modifications. Cells were washed twice and resuspended in complete RPMI 1640 medium at a cellular density of 4×10^8 cells ml^{-1}. Aliquots (100 µl) of the cell suspension were incubated with increasing concentrations of verapamil and cyclosporine-A, as well as GLIB, 30 min at RT; the cells were further incubated with 0.25 µM CAL-AM, 30 min at RT and centrifuged at 4 °C, 200*g*, 5 s. The supernatant was aspirated and the pellet was washed twice with 1 ml RPMI 1640 and resuspended in 200 µL cold RPMI 1640; the fluorescence signal was monitored on a Perkin Elmer Victor II spectrofluorimeter at 517 nm ($\lambda_{ex} = 494$ nm).

To calculate the IC_{50}, we determined the concentration of inhibitor needed for 50% inhibition of CAL-AM efflux using the following equation: calcein retention = $[F(\text{treated})/F(\text{untreated})] \times 100$, where F(treated) and F(untreated), represent the fluorescence detected in parental or resistant cells, respectively, at each concentration of inhibitor tested.

2.8. Results analysis

Statistical analysis was performed with the program GraphPad® 4.00. Growth curve experiments were repeated three times in duplicate and compared by non linear regression. A 95% interval of confidence (IC) is given when comparing inhibition of cell growth by the antimetabolites

2-MA and UL-2-DOG. Data on glucose utilization and ammonium production are expressed as means ± SEM of five independent experiments done in duplicate. Data on CAL-AM, CAL and *2-DOG kinetics are the result of at least three independent experiments done in quintuplicate; initial rates were analyzed by linear regression. Relationships between $V_{[CAL\text{-}AM]}$ and [CAL-AM]c, as well as between $V_{[CAL]}$ and $[CAL]_c$ were analyzed by linear regression. Slopes of best fit represented $K_{CAL\text{-}AM}$ and K_{CAL} constants, respectively. Data on CAL retention inhibition are the result of three independent experiments made in duplicate, and the IC_{50} is presented as mean ± SEM. Statistical differences between glucose utilization, ammonium production, *2-DOG transport, $K_{CAL\text{-}AM}$ and K_{CAL}, as well as CAL retention were evaluated by the Student *t* test.

3. Results

3.1. Glibenclamide resistance decreased glucose utilization in LTB^{160}

Under our experimental conditions, growth of LTB^{160} and LTB lasted 7–8 days. Like in NR(Gs) and NR(Gr) (Uzcategui et al., 2005), there were only minor growth differences between the two strains (Fig. 1, □, ■). Changes in glucose and ammonium in the culture media, an indication of glucose utilization and amino acid catabolism, were measured at the end of the exponential phase of growth. During their exponential growth LTB^{160} used 35.57 ± 0.40 mmol glucose 10^9 cells while LTB used 45.00 ± 0.70 mmol glucose 10^9 cells, ($p < 0.003$); LTB^{160} parasites produced 4.50 ± 0.20 mmol ammonium 10^9 cells during their exponential growth phase, while LTB parasites produced 4.40 ± 0.40 mmol ammonium 10^9 cells (not significant) during their exponential growth. In other words, LTB^{160} and LTB grow similarly, but GLIB-resistant *Leishmania* consume 25% less glucose, and similar amounts of ammonium than the wild-type cells.

3.2. Glibenclamide resistance decreased *2-DOG accumulation by LTB^{160}

To further understand the previous results, we compared the characteristics of *2-DOG cell accumulation by LTB^{160} and LTB. The rate of *2-DOG accumulation was linear for at least 180 s in LTB and LTB^{160}. The rate of uptake saturated afterwards and the cellular levels of *2-DOG reached a plateau. Incorporation of *2-DOG was measured during the first 120 s (Table 1). The acute exposure of LTB to GLIB decreased dramatically the velocity of *2-DOG accumulation ($p < 0.001$) (Table 1). However, the velocity of *2-DOG accumulation was 2- fold lower in LTB^{160} than in LTB ($p < 0.001$) when the GLIB-resistant strain was incubated in the presence of GLIB and 9- fold lower ($p < 0.001$) in LTB^{160} than in LTB when both cell lines were incubated in the absence of GLIB, (Table 1). D-Glucose, L-Glucose, or

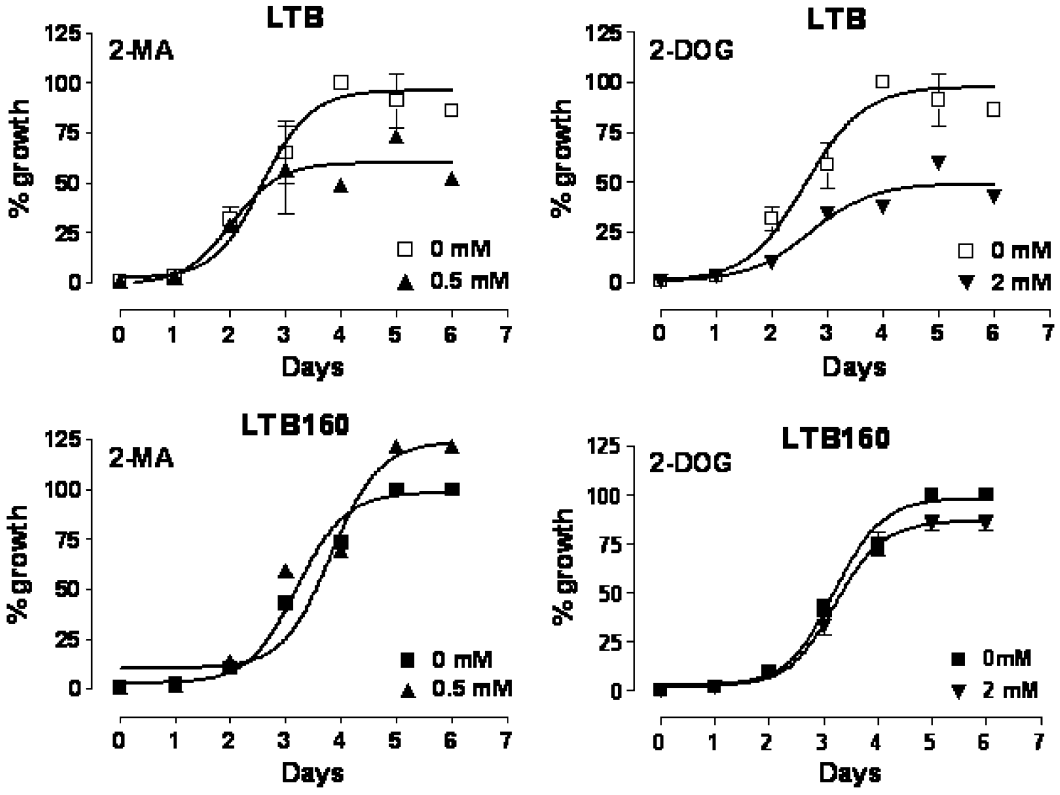

Fig. 1. Growth of LTB and LTB^{160} in the presence of 2-MA or 2-DOG. Parasites were seeded at 2×10^6 ml^{-1} alone or with 0.5 mM 2-MA or 2 mM 2-DOG. Parasite growth was then followed daily until day seven.

Table 1
Kinetics of glucose transport in LTB and LTB^{160}

	*2-DOG accumulation (mmol × 10^{-14} × cell^{-1} × s^{-1})		Inhibition of *2-DOG accumulation (mmol × 10^{-14} × cell^{-1} × s^{-1})		
	−GLIB	+GLIB	D-Glucose	L-Glucose	UL-2-DOG
LTB	11.04[a] ± 2.76	−0.18[a] ± 0.02	−17.81 ± 3.20	−11.48 ± 0.15	−12.51 ± 1.47
LTB^{160}	1.26[a] ± 0.26	5.65[a] ± 1.36	−16.55 ± 0.29	−11.13 ± 0.93	−13.63 ± 0.30

[a] $p = 0.001$.

UL-2-DOG inhibited *2-DOG accumulation with similar rates in both strains (Table 1). Of note, *2-DOG accumulation in LTB^{160} grown in the absence of GLIB for at least 6 months remained at 5.041 ± 0.125 mmol × 10^{-14} × cell^{-1} s^{-1}, thus demonstrating considerable stability of the decreased velocity of *2-DOG accumulation by GLIB-resistant parasites.

3.3. Glibenclamide resistance decreased sensitivity to 2-MA and 2-DOG of LTB^{160}

A decreased velocity of glucose accumulation appears to be a metabolic adaptation of drug-resistant *Leishmania*. To further analyze this metabolic adaptation we compared the sensitivity of LTB and LTB^{160} to the antimetabolites 2-MA and UL-2-DOG. GLIB-sensitive and -resistant parasites were grown in the absence and presence of various concentrations of 2-MA or UL-2-DOG. Herein we describe the results obtained with the antimetabolite concentration that inhibited 50% LTB growth. Indeed, a concentration of 0.5 mM 2-MA did not affect the growth of LTB^{160} but decreased the growth of LTB 50% (IC 36–63%) (Fig. 1). A concentration of 2 mM UL-2-DOG did not affect the growth of LTB^{160} but decreased the growth of LTB by 50% (IC 47–73%) (Fig. 1). We further evaluated if the inhibition of parasite growth was associated with changes in the cell glucose uptake and ammonium production. Importantly, inhibition of LTB growth by 2-MA was associated with a two fold increase in glucose uptake ($p = 0.008$) (Table 2), while inhibition of LTB growth by UL-2-DOG was associated with a 1.5 fold increase in glucose uptake ($p = 0.001$). Neither 2-MA nor 2-DOG inhibition of LTB growth affected the production of ammonium (Table 2); importantly, glucose uptake and ammonium production of LTB^{160} grown in the presence of the antimetabolites did not change (Table 2). These results indicate that GLIB-resistant LTB^{160} is less sensitive to the antimetabolites UL-2-DOG and 2-MA than GLIB-sensitive LTB and that the slower glucose uptake evidenced in this strain may be responsible for this decreased sensitivity.

3.4. Glibenclamide resistance induced the expression of ABC proteins in LTB^{160}

To evaluate the function of ABC transporters in *L. amazonensis*, LTB and LTB^{160} promastigotes were incubated with 0.5, 0.75, and 2 μM CAL-AM. The increase in fluorescence was measured against time. Herein we illustrate the experiments carried out with LTB^{160} at 0.75 μM CAL-AM (Fig. 2); similar profiles were obtained for the parental strain LTB (data not shown). After measuring CAL formation at each CAL-AM concentration we calculated the rate of CAL formation d[CAL]/dt against $[\text{CAL-AM}]_0$ (Fig. 3). A linear relationship was obtained between the former and the latter. Afterwards we determined the relationship between the rate of CAL-AM active efflux (differences between curve c and a, Fig. 3) and the intracellular CAL-AM concentration $[\text{CAL-AM}]_c$. The resulting plot, $V_{[\text{CAL-AM}]}$ as a function of $[\text{CAL-AM}]_c$ is given in Fig. 4A. A linear relationship was obtained within the concentration range studied. The slope of the line characterizes the efficiency of CAL-AM efflux or $K_{\text{CAL-AM}}$. The $K_{\text{CAL-AM}}$ for LTB^{160} [(1.1949 ± 0.1) 10^{-13} L s^{-1} cell^{-1}] was 3- fold higher ($p < 0.0001$) than that of LTB [(0.2965 ± 0.04) 10^{-13} L s^{-1} cell^{-1}]. The linear relationship obtained between the rate of CAL extrusion ($V_{[\text{CAL}]}$) and the intracellular CAL concentration ($[\text{CAL}]_c$) is presented in Fig. 4B. The slope K_{CAL} characterizes the efficiency of CAL extrusion. Only for LTB^{160} a K_{CAL} of (2.1226 ± 0.4) 10^{-16} L s^{-1} cell^{-1} was determined, the values for LTB were below detection threshold. These results demonstrate that *L. amazonensis* constitutively expresses ABC transporters that mobilize the neutral substrate CAL-AM, with higher efficiency in LTB^{160} than in LTB, and that GLIB resistance induces the expression of ABC proteins that transfers the anionic substrate CAL.

Table 2
Use of glucose and production of ammonium in LTB and LTB^{160} treated with 2-MA and UL-2-DOG

	Control (mmol glucose 10^{-9} cells)	2-MA (mmol glucose 10^{-9} cells)	UL-2-DOG (mmol glucose 10^{-9} cells)	Control (mmol ammonium 10^{-9} cells)	2-MA (mmol ammonium 10^{-9} cells)	UL-2-DOG (mmol ammonium 10^{-9} cells)
LTB	45.00[a,b] ± 0.70	83.00[a] ± 11.00	62.00[b] ± 3.17	4.40 ± 0.40	7.60 ± 1.60	6.00 ± 1.00
LTB^{160}	35.57 ± 0.40	40.60 ± 2.10	39.20 ± 2.40	4.50 ± 0.20	3.80 ± 0.30	5.20 ± 0.60

[a] $p = 0.008$.
[b] $p = 0.001$.

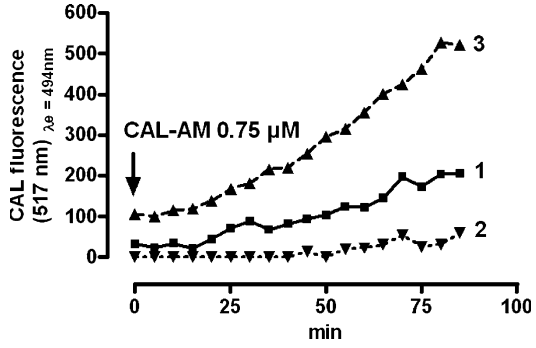

Fig. 2. Time course of CAL-AM uptake by LTB[160] promastigotes. CAL fluorescence was recorded at 517 nm as a function of time. CAL-AM (0.75 µM final concentration) was added at time zero. Intracellular CAL-AM conversion to CAL increases the fluorescence, and the fluorescent probe location is deduced from the experimental conditions of each curve. In curve 1 the transport system works under optimal conditions (energized cells, i.e., preincubated with 5 mM glucose), and both intracellular and extracellular CAL produce fluorescence. In curve 3 the transport system is inhibited (cells are preincubated 30 min with 10 mM sodium azide and without glucose), and the fluorescence remains within the cell. Finally in curve 2, cells were incubated simultaneously in the presence of glucose and 2 µM Co^2 which quenches the extracellular fluorescence; the recorded fluorescence represents the intracellular CAL. Differences between curves 1 and 3 estimate CAL-AM extrusion and differences between curves 1 and 2 estimate CAL extrusion from the cells (Essodaïgui et al., 1998, 1999).

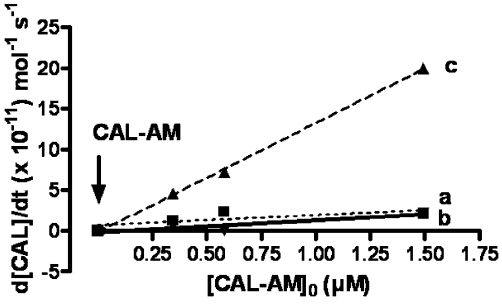

Fig. 3. Initial rates of CAL formation d[CAL]/dt by LTB[160] promastigotes. The initial rate of CAL formation has been plotted as a function of CAL-AM concentration in the extracellular medium. Cells were energized in the absence (curve a) and presence (curve b) of 2 µM Co^{2+}. Other cells were energy deprived (curve c). d[CAL]/dt was determined from curves as those displayed in Fig. 2 at 30 min (Essodaïgui et al., 1998, 1999).

3.5. ABC transporters from LTB[160] and LTB were sensitive to MRP inhibitors

Finally, we characterized the ABC transporters expressed in *L. amazonensis*. We evaluated cell CAL retention and its inhibition by competing substrates for P-gp and MRP, such as Cyclosporine-A and Verapamil, as well as GLIB. Basal CAL retention by LTB was 50% higher than by LTB[160] ($p < 0.05$). Furthermore, within the concentration range studied herein, Cyclosporine-A could not increase CAL retention in any strain. A concentration of 14.00 ± 4.00 µM and 128.00 ± 36.00 µM, respectively, of Verapamil and GLIB increased 50% CAL retention by LTB[160]; lower concentrations, that is, 3.00 ± 0.80 µM Verapamil and 60.00 ± 26.00 µM GLIB increased 50% CAL

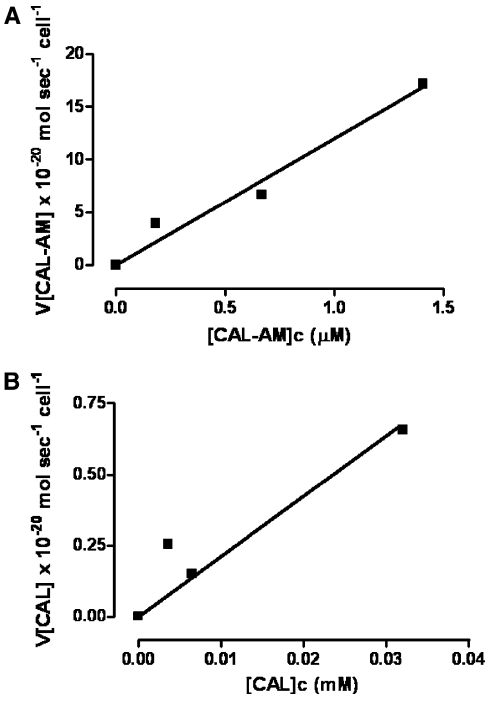

Fig. 4. Kinetics of CAL-AM and CAL active efflux in LTB[160]. (A) Rate of CAL-AM active extrusion $V_{[CAL\text{-}AM]}$ plotted as a function of $[CAL\text{-}AM]_c$. $V_{[CAL\text{-}AM]}$ is given at each extracellular CAL-AM concentration by differences between d[CAL]/dt and d[CAL]/dt (curves c and a, Fig. 3), and $[CAL\text{-}AM]_c$ was obtained by reading the value for $[CAL\text{-}AM]_c$ corresponding to d[CAL]/dt (curve a on curve c, Fig. 3). The slope of the linear relationship $K_{CAL\text{-}AM}$ represents CAL-AM flux efficiency. (B) Rate of CAL active extrusion $V_{[CAL]}$ plotted as a function of $[CAL]_c$. $[CAL]_c$ was determined from the fluorescence signal in the presence of Co^{2+} and estimating the cell volume as 2×10^{-14} L (Bandyopadhyay et al., 1991; Essodaïgui et al., 1998, 1999). K_{CAL} characterizes CAL efflux efficiency.

retention by LTB (Table 3). These results further demonstrate that the drug transport systems expressed in *Leishmania* display different efficiencies and are sensitive to MRP inhibitors like verapamil and GLIB, but not to the P-gp inhibitor cyclosporine-A.

4. Discussion

Cellular events that occur along with *Leishmania* drug resistance include the overexpression of ABC proteins that modulate the efflux or intracellular trafficking of chemotherapeutic agents (Jones and George, 2005; Ouellette et al., 2004). Furthermore, the development of drug resistance

Table 3
Inhibition of CAL retention by Cyclosporine-A, Verapamil and Glibenclamide in LTB and LTB[160]

Inhibitor	µM concentration for 50% inhibition of CAL retention	
	LTB	LTB[160]
Cyclosporine-A	n.d.[a]	n.d.[a]
Verapamil	3.00 ± 0.80	14.00 ± 4.00
Glibenclamide	60.00 ± 26.00	128.00 ± 36.00

n.d., non detected.
[a] concentrations up to 100 µM cyclosporine-A were used.

induces alteration of other biochemical and physiological parameters such as changes in xenobiotics conjugation and traffic, cytoskeleton phosphorylation, membrane microviscosity and composition, metacyclogenesis and infectivity, and mitochondrial gene expression (reviewed by Ponte-Sucre, 2003), but the associated metabolic changes are poorly understood.

In mammalian drug-resistant cells, metabolic strategies involve increased glucose transport and metabolism and increased oxygen consumption (Harper et al., 2002). Previous results suggest that drug-resistant *Leishmania* use other metabolic strategies (García et al., 2000; Uzcategui et al., 2005). Herein we demonstrate that although a decreased growth rate was not evident in the resistant strain (LTB^{160}), slower glucose uptake, and decreased sensitivity to antimetabolites might constitute general mechanisms that underlie drug resistance in this parasite.

In a Venezuelan *Leishmania*, selection for resistance to GLIB is accompanied by decreased glucose utilization, glucose transporter expression (Uzcategui et al., 2005) and activity of enzymes such as HK and, especially, PGI and PK (García et al., 2000; Uzcategui et al., 2005). Here, we demonstrate that exponentially growing LTB^{160} utilize less glucose than LTB, using transporters that share characteristics with LTB, *L. major* (C. Machuca, unpublished), and most *Leishmania* species (Burchmore and Hart, 1995; Schaeffer et al., 1974; Zilberstein and Dwyer, 1984).

In mammalian cells GLIB stimulates glucose accumulation (Farese et al., 1991; Jacobs et al., 1989; Rogers et al., 1987) and stabilizes glucose transporters at the plasma membrane (Tsiani et al., 1995); surprisingly, acute exposure of *L. major* (C. Machuca, unpublished) or LTB (present study) to GLIB dramatically decreases glucose accumulation. Furthermore, glucose accumulation was 9- fold slower in LTB^{160} than in LTB, and glucose accumulation in LTB^{160} exposed to GLIB remained 2- fold slower than in sensitive cells not exposed to GLIB. We do not have a straightforward explanation for these results that strongly suggest that the action of GLIB in *Leishmania* differs from that in mammalian cells.

Drug-resistant *Leishmania* maintains normal growth (herein and Uzcategui et al., 2005), although functional characters associated with an infective phenotype are reduced (Silva et al., 2004). Decreased infectivity occurs in drug-resistant *L. donovani*, *Leishmania guyanensis* and *L. mexicana amazonensis* (Gazola et al., 2001; Prasad et al., 2000; Silva et al., 2004), and *L. mexicana* mutants for glucose transport (Δ*lmgt*) do not sustain infection in murine models (Burchmore et al., 2003); full viability is partially restored in Δ*lmgt* mutants by rescue with the glucose transporter *LmGT3*. As sugars are fundamental for the development of infective *Leishmania* (Killick-Kendrick, 1979) and scavenging of sparse glucose may be fundamental for amastigote survival (Burchmore et al., 2003), our results strongly suggest that the slower glucose accumulation in drug-resistant *Leishmania* may render parasites with a competitive disadvantage for causing infection in the host.

The antimetabolites 2-MA (an inhibitor of acyl-CoA dehydrogenase, Bauché et al., 1981) and UL-2-DOG (a non-metabolizable analogue of glucose, Brown, 1962) did not affect the growth of LTB^{160} at concentrations that inhibited 50% the growth of LTB. These antimetabolites decrease cellular glucose and amino acids availability; a significant increase of glucose uptake was observed in LTB but not in LTB^{160} treated with the antimetabolites. Although the inhibition of *2-DOG accumulation by D-glucose, L-glucose and UL-2-DOG in both LTB and LTB^{160} with comparable rates is a result difficult to explain; the slower glucose uptake herein described for the first time in LTB^{160} may be responsible for this decreased sensitivity.

Finally, we evaluated the function of ABC transporters for neutral and organic acids by measuring the rate of CAL-AM and CAL extrusion and retention in LTB^{160} and LTB. Our data demonstrate for the first time that energy dependent CAL-AM efflux systems constitutively expressed in *L. amazonensis* display similar rates as in *L. major* (C. Machuca, unpublished) *L. mexicana*, *L.* (*V.*) *brasiliensis*, and *L. guyanensis* (Essodaïgui et al., 1999). Additionally, our data demonstrate that the CAL-AM transport systems in LTB^{160} are faster than in LTB and that an energy dependent CAL transport system 10^3 fold less efficient than the CAL-AM transport systems (Saengkhae et al., 2003) is expressed only in LTB^{160}.

These results strongly suggest that a decreased accumulation of CAL-AM and CAL is associated with an increased expression of ABC transporters in *L. amazonensis*, as has been described in several *Leishmania* species (Dodge et al., 2004; Essodaïgui et al., 1999). Although the present data do not allow tracing the subcellular localization of the CAL-AM transporter as has been done for *Leishmania enrietti* (Dodge et al., 2004) experiments in this direction would be very interesting to perform.

Similar to what has been described for PRP1 from *L. major* (Coelho et al., 2003), Cyclosporine-A, a P-gp inhibitor (Bonafonte et al., 2004; Ford and Hait, 1990; Foxwell et al., 1989), did not inhibit CAL retention, and a result not easy to reconcile with a drug transport mechanism through P-gp like transporters (Jones and George, 2005). On the contrary, Verapamil ($LTB < LTB^{160}$) and GLIB ($LTB < LTB^{160}$), that interfere with MRP-1-like organic anion transporters (Loe et al., 2000; Tsuruo, 1983) in parasites (Bonafonte et al., 2004), yeast and plants (Forestier et al., 2003) as well as human tissues (Lamensdorf et al., 2000; Payen et al., 2001; Salerno et al., 2004) herein inhibited the CAL retention. Importantly, these data confirm the constitutive expression in *Leishmania* sp. of systems that transport neutral non-metabolized compounds (Essodaïgui et al., 1998, 1999) and the up-regulation by drug resistance of MRP-1 like systems that transfer anionic compounds. Additionally they prove for the first time that these systems express similar sensitivity for verapamil and GLIB.

Altogether, these results suggest that the metabolic strategies used by drug-resistant *Leishmania* differ from those described for mammalian resistant cells. Although a

decreased growth rate was not evident in the resistant strain, the slower glucose uptake, together with the decreased activity of enzymes with specific activities that have been described to vary in a coordinated way with changes in growth rates, the absence of modulation on ATP production produced by drug resistance in *Leishmania* (Singh and Lee, 1999) and the decreased sensitivity of resistant parasites to the antimetabolites could represent a fitness cost (decreased virulence), and may constitute general mechanisms that underlie drug resistance in this parasite. Furthermore, up-regulation of alternative metabolic routes for other substrates (Uzcategui et al., 2005) may be an advantage for the growth of resistant cells. Understanding these mechanisms may be helpful for identifying strategies to circumvent *Leishmania* drug resistance and designing alternative therapies for the successful treatment of leishmaniasis.

Acknowledgments

This project was supported by the Universidad Central de Venezuela Faculty of Medicine grant, FAC-MED, 000054/2000 and Council for Research grant CDCH, PI-09.33.5520.2003, CDCH PI-09.00.6027.2005, CDCH, A-09.005783.2005. We are grateful to Dr. Lee Schnurr for supplying us with the LTB *Leishmania* strain. The Perkin Elmer Victor II Spectrofluorimeter was a donation from the Alexander von Humboldt Foundation, Germany.

References

Bauché, F., Sabourault, D., Nordmann, J., Nordmann, R., 1981. 2-Mercaptoacetate administration depresses the β-oxidation pathway through an inhibition of long-chain acyl CoA dehydrogenase activity. Biochemical Journal 196, 803–807.

Bandyopadhyay, P., Ghosh, D.K., De, A., Ghosh, K.N., Chaudhuri, P.P., Das, P., Bhattacharya, A., 1991. Metacyclogenesis of *Leishmania spp*: species-specific in vitro transformation, complement resistance, and cell surface carbohydrate and protein profiles. Journal of Parasitology 77, 411–416.

Becq, F., Hamon, Y., Bajetto, A., Gola, M., Verrier, B., Chimini, G., 1997. ABC1, an ATP binding cassette transporter required for phagocytosis of apoptotic cells, generates a regulated anion flux after expression in *Xenopus laevis* oocytes. Journal of Biological Chemistry 272, 2695–2699.

Bonafonte, M.T., Romagnoli, P.A., McNair, N., Shaw, A.P., Scanlon, M., Leitch, G.J., Mead, J.R., 2004. *Cryptosporidium parvum*: effect of multidrug reversing agents on the expression and function of ATP-binding cassette transporters. Experimental Parasitology 106, 126–134.

Borst, P., Ouellette, M., 1995. New mechanisms of drug resistance in parasitic protozoa. Annual Review of Microbiology 49, 427–460.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 246–254.

Brown, J., 1962. Effects of 2-deoxyglucose on carbohydrate metabolism: review of the literature and studies in the rat. Metabolism 11, 1098–10112.

Burchmore, R.J., Hart, D.T., 1995. Glucose transport in amastigotes and promastigotes of *Leishmania mexicana*. Molecular and Biochemical Parasitology 74, 76–86.

Burchmore, R.J., Rodriguez-Contreras, D., McBride, K., Merkel, P., Barrett, M.P., Modi, G., Sacks, D., Landfear, S.M., 2003. Genetic characterization of glucose transporter function in *Leishmania mexicana*. Proceedings of the National Academy of Sciences, United States of America 100, 3901–3906.

Coelho, A.C., Beverley, S.M., Cotrim, P.C., 2003. Functional genetic identification of PRP1 an ABC transporter superfamily member conferring pentamidine resistance in *Leishmania major*. Molecular and Biochemical Parasitology 130, 83–90.

Cole, S.P.C., Bhardwaj, G., Gerlach, J.H., Mackie, J.E., Grant, C.E., Almquist, A.J., Stewart, A.J., Kurz, E.U., Duncan, A.M.V., Deeley, R.G., 1992. Overexpression of a transporter gene in a multidrug resistant human lung cancer cell line. Science 258, 1650–1654.

Conseil, G., Deeley, R.G., Cole, S.P., 2005. Role of two adjacent cytoplasmic tyrosine residues in MRP1 (ABCC1) transport activity and sensitivity to sulfonylureas. Biochemical Pharmacology 69, 451–461.

Chow, L.M., Wong, A.K., Ullman, B., Wirth, D.F., 1993. Cloning and functional analysis of an extrachromosomally amplified multidrug resistance-like gene in *Leishmania enriettii*. Molecular and Biochemical Parasitology 60, 195–208.

Dodge, M.A., Waller, R.F., Chow, L.M., Zaman, M.M., Cotton, L.M., McConville, M.J., Wirth, D.F., 2004. Localization and activity of multidrug resistance protein 1 in the secretory pathway of *Leishmania* parasites. Molecular Microbiology 51, 1563–1575.

Essodaïgui, M., Broxterman, H., Garnier-Suillerot, A., 1998. Kinetic analysis of calcein and calcein acetoxymethyl ester efflux mediated by the multidrug resistance protein and p-glycoprotein. Biochemistry 37, 2243–2250.

Essodaïgui, M., Freezard, F., Moreira, E.S.A., Dagger, F., Garnier-Suillerot, A., 1999. Energy-dependent efflux from *Leishmania* promastigotes of substrates of the mammalian multidrug resistance pumps. Molecular and Biochemical Parasitology 100, 73–84.

Farese, R.V., Ishizuka, T., Standaert, M.L., Cooper, D.R., 1991. Sulfonylureas activate glucose transport and protein kinase C in rat adipocytes. Metabolism 40, 196–200.

Ford, J.M., Hait, W.N., 1990. Pharmacology of drugs that alter multidrug resistance in cancer. Pharmacological Reviews 42, 155–199.

Forestier, C., Frangne, N., Eggmann, T., Klein, M., 2003. Differential sensitivity of plant and yeast MRP (ABCC)-mediated organic anion transport processes towards sulfonylureas. FEBS Letters 554, 23–29.

Foxwell, B.M., Mackie, A., Ling, V., Ryffel, B., 1989. Identification of the multidrug resistance-related P-glycoprotein as a cyclosporine binding protein. Molecular Pharmacology 36, 543–546.

García, N., Figarella, K., Mendoza-León, A., Ponte-Sucre, A., 2000. Changes in the infectivity, pyruvate kinase and acid phosphatase activity and P-glycoprotein expression in glibenclamide resistant *Leishmania mexicana*. Parasitology Research 86, 899–904.

Gazola, K.C., Ferreira, A.V., Anacleto, C., Michalik, M.S., Andrade, A., Moreira, E.S., 2001. Cell surface carbohydrates and in vivo infectivity of glucantime sensitive and resistant *Leishmania (viannia) guyanensis* cell lines. Parasitology Research 87, 935–940.

Golstein, P.E., Boom, A., Van Geffel, J., Jacobs, P., Maseret, B., Beauwens, R., 1999. P-glycoprotein inhibition by glibenclamide and related compounds. Pfluggers Archives 437, 652–660.

Gottesman, M.M., Hrycyna, C.A., Schoenlein, P.V., Germann, U.A., Pastan, I., 1995. Genetic analysis of the multidrug transporter. Annual Review of Genetics 29, 607–649.

Hamon, Y., Luciani, M.F., Becq, F., Verrier, B., Rubartelli, A., Chimini, G., 1997. Interleukin-1 beta secretion is impaired by inhibitors of the ATP binding cassette transporter, ABC1. Blood 90, 2911–2915.

Harper, M.E., Antoniou, A., Villalobos-Menuey, E., Russo, A., Trauger, R., Vendemelio, M., George, A., Bartholomew, R., Carlo, D., Shaikh, A., Kupperman, J., Rogers, J.R., Gibbs, G.L., Leahy, J.L., Camley, R.E., Melamede, R., Newell, K., 2002. Characterization of a novel metabolic strategy used by drug-resistant tumor cells. FASEB Journal 16, 1550–1557.

Henderson, D.M., Sifri, C.D., Rodgers, M., Wirth, D.F., Hendrickson, N., Ullman, B., 1992. Multidrug resistance in *Leishmania donovani* is conferred by amplification of a gene homologous to the mammalian *mdr1* gene. Molecular Cell Biology 12, 2855–2865.

Higgins, C.F., 1992. ABC transporters: from microorganisms to man. Annual Review of Cell Biology 8, 67–113.

Inagaki, N., Gonoi, T., Clement IV, J.P., Namba, N., Inazawa, J., Gonzalez, G., Aguilar-Bryan, L., Seino, S., Bryan, J., 1995. Reconstitution of IK_{ATP}: an inward rectifier sub-unit plus the sulfonylurea receptor. Science 270, 1166–1169.

Jacobs, D.B., Hayes, G.R., Lockwood, D.H., 1989. In vitro effects of sulfonylurea on glucose transport and translocation of glucose transporters in adipocytes from streptozocin-induced diabetic rats. Diabetes 38, 205–211.

Jones, P.M., George, A.M., 2005. Multidrug resistance in parasites: ABC transporters, P-glycoproteins and molecular modelling. International Journal of Parasitology 35, 555–566.

Juliano, R.L., Ling, V., 1976. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. Biochemical and Biophysical Acta 455, 152–162.

Katakura, K., Iwanami, M., Ohtomo, H., Fujise, H., Hashiguchi, Y., 1999. Structural and functional analysis of the LaMDR1 multidrug resistance gene in *Leishmania amazonensis*. Biochemical and Biophysical Research Communication 255, 289–294.

Killick-Kendrick, R., 1979. Biology *of Leishmania* in phlebotomine sand flies. In: Lumsden, W.H.R., Evans, D.A. (Eds.), Biology of Kinetoplastida, Vol. 2. Academic Press, London, pp. 395–460.

Lamensdorf, I., Hrycyna, C., He, L.P., Nechushtan, A., Tjurmina, O., Harvey-White, J., Eisenhofer, G., Rojas, E., Kopin, I.J., 2000. Acidic dopamine metabolites are actively extruded from PC12 cells by a novel sulfonylurea-sensitive transporter. Naunyn Schmiedebergs Archives Pharmacology 361, 654–664.

Légaré, D., Hettema, E., Ouellette, M., 1994. The P-glycoprotein-related gene family in *Leishmania*. Molecular and Biochemical Parasitology 68, 81–91.

Légaré, D., Cayer, S., Singh, A.K., Richard, D., Papadopoulou, B., Ouellette, M., 2001. ABC proteins in *Leishmania*. Journal of Bioenergetics and Biomembranes 33, 469–474.

Lee, E.K., Kwon, M., Ko, J.H., Yi, H., Hwang, M.G., Chang, S., Cho, M.H., 2004. Binding of sulfonylurea by AtMRP5, an *Arabidopsis* multidrug resistance-related protein that functions in salt tolerance. Plant Physiology 134, 528–538.

Loe, D.W., Deeley, R.G., Cole, S.P., 2000. Verapamil stimulates glutathione transport by the 190-kDa multidrug resistance protein 1 (MRP1). Journal of Pharmacology and Experimental Therapeutics 293, 530–538.

Lyon, R.C., Cohen, J.S., Faustino, P.J., Megnin, F., Myers, C.E., 1988. Glucose metabolism in drug-sensitive and drug-resistant human breast cancer cells monitored by magnetic resonance spectroscopy. Cancer Research 48, 870–877.

Martell, R.L., Slapak, C.A., Levy, S.B., 1997. Effect of glucose transport inhibitors on vincristine efflux in multidrug resistant murine erythroleukaemia cells overexpressing the multidrug resistance associated protein (MRP) and two glucose transport proteins GLUT1 and GLUT3. British Journal of Cancer 75, 161–168.

Ouellette, M., Fase-Fowler, F., Borst, P., 1990. The amplified H circle of methotrexate-resistant *Leishmania tarentolae* contains a novel P-glycoprotein gene. The EMBO Journal 9, 1027–1033.

Ouellette, M., Drummelsmith, J., Papadopolou, B., 2004. Leishmaniasis: drugs in the clinic, resistance and new developments. Drug Resistance Update 7, 257–266.

Payen, L., Delugin, L., Courtois, A., Trinquart, Y., Guillouzo, A., Fardel, O., 2001. The sulphonylurea glibenclamide inhibits multidrug resistance protein (MRP1) activity in human lung cancer cells. British Journal of Pharmacology 132, 778–784.

Ponte-Sucre, A., Campos, Y., Vázquez, J., Moll, H., Mendoza-León, A., 1997. Sensitivity of *Leishmania* spp. to glibenclamide and 4-aminopiridine: a tool for the study of drug resistance development. Memórias Instituto Oswaldo Cruz 92, 601–606.

Ponte-Sucre, A., Campos, Y., Fernández, M., Moll, H., Mendoza-León, A., 1998. *Leishmania sp.*: Growth and survival are impaired by ion channel blockers. Experimental Parasitology 88, 11–19.

Ponte-Sucre, A., 2003. Physiological consequences of *Leishmania* drug resistance. Kinetopastids Biology and Disease, 2, 14 (http://www.kinetoplastids.com/home/).

Prasad, V., Kumar, S.S., Dey, C., 2000. Resistance to arsenite modulates levels of α-tubulin and sensitivity to paclitaxel in *Leishmania donovani*. Parasitology Research 86, 838–842.

Rogers, B.J., Standaert, M.L., Pollet, R.J., 1987. Direct effects of sulfonylurea agents on glucose transport in the BC3H-1 myocyte. Diabetes 36, 1292–1296.

Saengkhae, C., Loetchutinat, C., Garnier-Suillerot, A., 2003. Kinetic analysis of fluorescein and dihydrofluorescein effluxes in tumour cells expressing the multidrug resistance protein, MRP1. Biochemical Pharmacology 65, 677–969.

Salerno, M., Loechariyakul, P., Saengkhae, C., Garnier-Suillerot, A., 2004. Relation between the ability of some compounds to modulate the MRP1-mediated efflux of glutathione and to inhibit the MRPl-mediated efflux of daunorubicin. Biochemical Pharmacology 68, 2159–2165.

Seyfang, A., Duszenko, M., 1991. Specificity of glucose transport in *Trypanosoma brucei*. Effective inhibition by phloretin and cytochalasin B. European Journal of Biochemistry 202, 191–196.

Schaeffer, F.W., Martin, E., Mukkada, A.J., 1974. The glucose transport system in *Leishmania tropica* promastigotes. Journal of Protozoology 21, 592–596.

Schultz, B.D., Deroos, A.D., Venglarik, C.J., Singh, A.K., Frizzell, R.A., Bridges, R.J., 1996. Glibenclamide blockade of CFTR chloride channels. American Journal of Physiology 271, L192–L200.

Silva, N., Camacho, N., Figarella, K., Ponte-Sucre, A., 2004. Cell differentiation and infectivity of *Leishmania mexicana* are inhibited in a strain resistant to an ABC-transporter blocker. Parasitology 128, 629–634.

Singh, A.K., Lee, S.T., 1999. Status of respiration and ATP content in arsenite resistant *Leishmania mexicana amazonensis*. Microbial Pathogenesiss 26, 171–174.

ter Kuile, B.H., Opperdoes, F.R., 1993. Uptake and turnover of glucose in *Leishmania donovani*. Molecular and Biochemical Parasitology 60, 313–321.

ter Kuile, B.H., 1999. Regulation and adaptation of glucose metabolism of the parasitic protist *Leishmania donovani* at the enzyme and mRNA level. Journal of Bacteriology 181, 4863–4872.

Tielens, A.G.M., Van Hellemond, J.J., 1998. Differences in energy metabolism between trypanosomatidae. Parasitology Today 14, 265–271.

Tsiani, E., Ramlal, T., Leiter, L.A., Klip, A., Fantus, I.G., 1995. Stimulation of glucose uptake and increased plasma membrane content of glucose transporters in L6 skeletal muscle cells by the sulfonylureas gliclazide and glyburide. Endocrinology 136, 2505–2512.

Tsuruo, T., 1983. Reversal of acquired resistance to vinca alkaloids and anthracycline antibiotics. Cancer Treatment Reports 67, 889–894.

Uzcategui, N.L., Figarella, K., Camacho, N., Ponte-Sucre, A., 2005. Substrate preferences and glucose uptake in glibenclamide-resistant *Leishmania* parasites. Comparative and Biochemical Physiology 140, 395–402.

Vera, J.C., Castillo, G.R., Rosen, O., 1991. A possible role for a mammalian facilitative hexose transporter in the development of resistance to drugs. Molecular Cell Bilogy 11, 3407–3418.

Zilberstein, D., Dwyer, D.M., 1984. Glucose transport in *Leishmania donovani* promastigotes. Molecular and Biochemical Parasitology 12, 327–334.