Correlation between glucose uptake and membrane potential in Leishmania *parasites isolated from DCL patients with therapeutic failure: a proof of concept*

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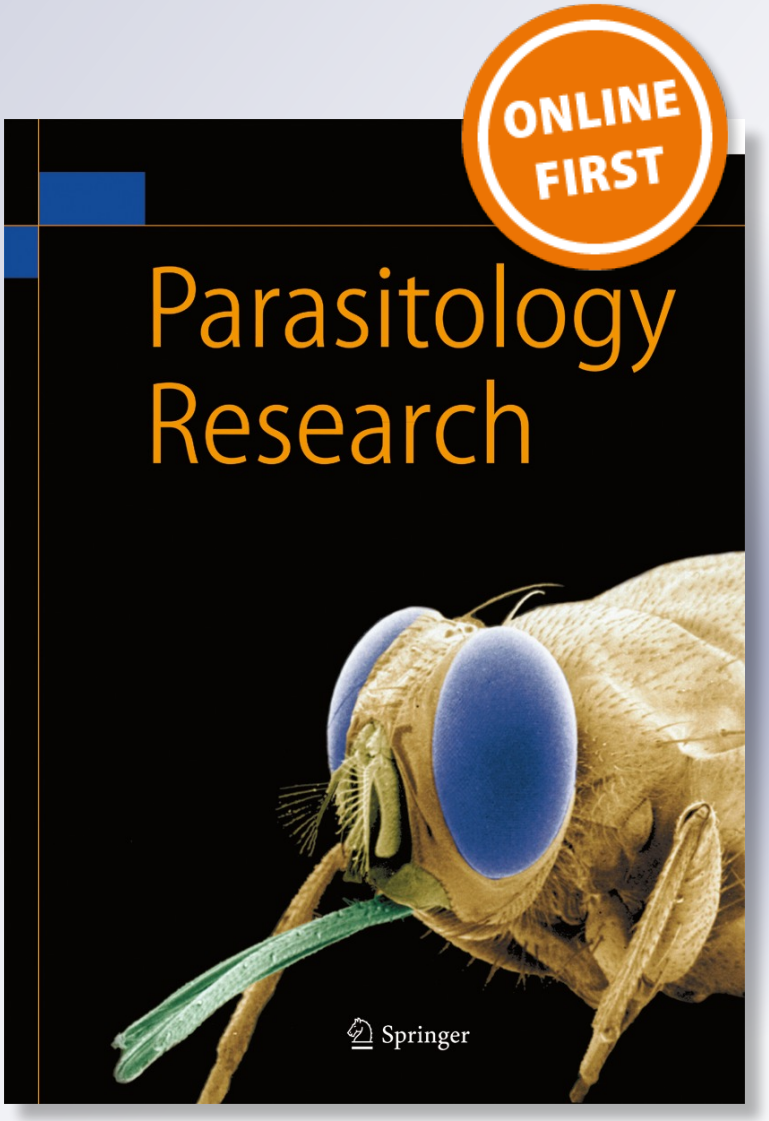

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ORIGINAL PAPER

Correlation between glucose uptake and membrane potential in *Leishmania* parasites isolated from DCL patients with therapeutic failure: a proof of concept

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Abstract Besides infection with drug-resistant parasites, therapeutic failure in leishmaniasis may be caused by altered drug pharmacokinetics, re-infection, and host immunologic compromise. Our aim has been to evaluate if relapses that occur in patients suffering from diffuse cutaneous leishmaniasis (DCL) associate with changes in the fitness of infecting organisms. Therefore, in isolates from patients suffering DCL, we correlated glucose uptake and plasma membrane potential and compared the results with those obtained from reference strains. The data demonstrate that *Leishmania* parasites causing DCL incorporate glucose at an efficient rate, albeit without significant changes in the plasma membrane potential as their corresponding reference strains. The isolate that did not change its accumulation rate of glucose compared to its reference strain expressed a less polarized membrane potential that was insensitive to mitochondrial inhibitors, suggesting a metabolic dysfunction that may result in glycolysis being the main source of ATP. The results constitute a proof of concept that indicates that parasites causing DCL adapted well to drug pressure and expressed an increased fitness. That is, that in *Leishmania mexicana* and *Leishmania amazonensis*, parasites isolated from DCL patients, a strong modification of the parasite physiology might occur. As consequences, the parasites adapted well to drug pressure, increased their fitness, and they had an efficient glucose uptake rate albeit not significant changes in membrane potential as their corresponding reference strains. Further validation of the concepts herein established and whether or not the third isolate corresponds with a drug-resistant phenotype need to be demonstrated at the genetic level.

Keywords Diffuse cutaneous leishmaniasis · Drug resistance markers · Glucose uptake · *Leishmania* · Membrane potential

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Introduction

Success of leishmaniasis chemotherapy depends on patient nutritional and immune status, severity of disease, whether the patient lives in an endemic area, and finally, the infecting *Leishmania* species. Classical treatment schemes include pentavalent antimony compounds such as sodium stibogluconate (Pentostam®) and meglumine antimoniate (Glucantime®); alternatively, the diamine pentamidine and the polyene antibiotic amphotericin-B, introduced in 1952 and 1960, respectively, are used as second-line drugs. Since 2000, the alkylphospholipid Miltefosine® is prescribed especially for visceral leishmaniasis. In Venezuela, since 2000, immunotherapy, either alone or in combination with Glucantime, is used for the treatment of the disease (Zerpa and Convit 2009; Maes et al. 2013).

The use of most of these drugs is challenging due to adverse effects, high cost, disadvantage of parenteral administration, duration of treatment (several weeks), and contraindication for pregnant women. Additionally, some patients do not respond to the drugs, and therapeutic failure may occur; finally, patients suffering from diffuse cutaneous leishmaniasis (DCL) have frequent relapses to any form of chemotherapy (Becker et al. 1999; Zerpa and Convit 2009; Goto and

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Lindoso 2010). Causes of treatment failure may also be related to an infection with resistant and/or tolerant parasites (Aït-Oudhia et al. 2011; Maes et al. 2013). This all means that parasites adapt to drug pressure and change their fitness to survive in the presence of this additional stress (Vanaerschot et al. 2010; Ponte-Sucre et al. 2013).

Chemoresistance has been defined as a decrease in drug efficacy in a population of previously susceptible organisms, assuming that the initial susceptibility is known, a fact not always fulfilled for *Leishmania*. In mammalian cells, increased expression of ATP-binding cassette (ABC) membrane transporters normally correlates with drug resistance. ABC membrane transporters indirectly, but dramatically, decrease drug access to the cellular or molecular target. Additionally, less accumulation of drug as a result of multidrug resistance transporter overexpression might be the consequence of changes in pH, V_m, and/or translocation and intracellular retention of weak bases (Roepe and Martiney 1999; Cadek et al. 2004). In *Leishmania*, chemoresistance has also been associated with decreased cellular accumulation of a drug as a consequence of the increased expression of ABC membrane transporters. However, epigenetic changes involving additional biochemical processes, which might alter the cell physiology, have also been described. Therefore, coexistence of multiple processes contributes to the preservation of chemoresistant phenotypes through mechanisms that guarantee parasite adaptation and survival in the adverse conditions produced by the continuous presence of drugs (Ponte-Sucre et al. 2013).

DCL is a rare entity within the spectrum of leishmaniasis. Over 100 cases have been described in Venezuela (De Lima et al. 2010). It is caused either by *Leishmania amazonensis* or *Leishmania mexicana*. The inclusion criteria comprise a poor response to antimonials with frequent relapses and a negative response to leishmanin (Zerpa and Convit 2009). Our aim has been to evaluate if relapses that occur in DCL patients associate with adaptation and fitness changes in the infecting parasites. Successful isolation and preservation of parasites from patients suffering DCL is rather challenging; however, we obtained three of them, stabilized their growth, and evaluated and correlated the rate of glucose incorporation with the cell membrane potential (V_m). We also compared the results with those obtained from reference strains. Two of the three isolates tested accumulated glucose at higher rates than reference strains, without dramatic changes in their V_m. The third isolate did not increase its glucose accumulation; interestingly, it had a more depolarized V_m than its corresponding reference strain. These results constitute a proof of concept suggesting that *Leishmania* parasites causing DCL may adapt well to drug pressure. This may also indicate an increase in their fitness as has been demonstrated in *Leishmania donovani* parasites isolated from patients suffering from Kala-azar, and not responding to the antimonial treatment (Vanaerschot et al. 2010). Further validation of the concepts herein established and whether or not the third isolate expresses a drug-resistant phenotype need to be demonstrated at the genetic level.

Materials and methods

Materials and instruments

The colorimetric method used to determine glucose (4-aminophenazone + phenol) was purchased from Human (Wiesbaden, Germany). Glucose concentrations were determined in a Spectronic (Milton Roy Company, USA). Plasma membrane potential (V_m) was determined by the use of the fluorescent compound bis-(1,3-dibutylbarbituric acid) trimethine oxonol (bisoxonol, Molecular Probes, USA). To determine V_m-specific fluorescence, changes were assessed in a Wallac Victor 2 Spectrofluorimeter (Perkin Elmer, Finland). Other reagents and solvents were purchased from Sigma-Aldrich, USA.

Parasites

Reference strains certified by the World Health Organization and used herein were: *L. amazonensis* (MHOM/BR/77/LTB0016) and *Leishmania major* MHOM/IL/81 donated by Dr. Lionel Schnur, Hebrew University-Hadassah Medical School, Jerusalem and *L. major* MHOM/SU/73/5ASKH and *L. mexicana* (MHOM/BR/82/Bel21). *L. major* PTR MHOM/IL/80/Friedlin/PTR is a cosmid transfected strain containing PRP1, an ABC transporter conferring pentamidine resistance in *L. major* (Coelho et al. 2003). *Leishmania* strains were maintained at 26 °C in semisolid blood agar supplemented with glucose–NaCl medium (glucose 1.5 %, NaCl 0.85 %, weight/volume).

Parasites isolated from lesions were obtained from three patients suffering DCL that regularly attended the Dermatology Department, Institute of Biomedicine, MPPS-UCV, Venezuela. The patients did not react to the treatment and had frequent relapses with Glucantime®. The following codes were assigned to the isolates: *L. mexicana* MHOM/VE/1996/ZC (VE96ZC), *L. amazonensis* MHOM/VE/1998/MR (VE98MR), and *L. amazonensis* MHOM/VE/2000/MM (VE2000MM). To minimize changes in their phenotype, the parasites were maintained in liquid nitrogen until use. The cells were then thawed and cultured and further maintained, at 26 °C in blood agar–glucose semisolid media–glucose medium until stable growth.

Glucose transport in parasites isolated from DCL patients

This study defines the rate of glucose incorporation to the cell regardless of subsequent metabolism (Machuca et al. 2006). We assessed the rate of glucose utilization by a modified

colorimetric method developed by Teuscher and Richterich (1971). Washed cells were re-suspended (5×10^8 cells ml^{-1}) in Hepes–glucose (132 mM NaCl; 3.5 mM KCl; 1 mM $CaCl_2$; 0.5 mM $MgCl_2$; 5 mM glucose in 10 mM Hepes, pH 7.4), and 100-μl aliquots were distributed into Eppendorf tubes (in quadruplicate). The tubes were incubated at 27 °C in a shaking bath. The first set of tubes was removed from the bath at minute 5; the remaining groups were removed every 10 min to complete 1 h. The reaction was stopped by centrifuging the tubes 30 s at 13,000×*g* at room temperature (RT). The supernatant (50 μl) was placed in new Eppendorf tubes, and 70 % perchloric acid (2.5 μl) was added. The tubes were stored at 4 °C until glucose determination. Calibration curves were performed in the range of 0 to 5 mM glucose. Glucose was determined as follows: To each sample (20 μl), 2 ml of the color reagent was added. After vortexing, the tubes were incubated 10 min at RT. Then, the absorbance was measured at 500 nm.

Membrane potential

To assess the cell membrane potential (V_m), bisoxonol was used. We evaluated the V_m with a methodology as described herein. Herein we aimed to evaluate parasite V_m of promastigote reference strains and isolates with a noninvasive fluorescent technique designed by Vieira et al. (1995) optimized in our laboratory and used for several parasite species (*L. donovani*, *L. major*, *Trypanosoma cruzi*, and *Giardia intestinalis* (Van Der Heyden and Docampo 2002; Biagini et al. 2000; Glaser et al. 1992)).

Calibration curves for V_m

Calibration curves for V_m in each strain of *Leishmania* were determined in the presence of increasing concentrations of potassium chloride (KCl). Aliquots of 200 μl (1×10^7 cells ml^{-1}) were resuspended in Hepes–*N*-methyl glucamine (NMGCl) buffer (Hepes 10 mM, NMGCl 140 mM, $MgCl_2$ 0.5 mM, glucose 11 mM, pH 7.3) and maintained at RT. The aliquots were incubated in darkness with valinomycin 1 μM, bisoxonol 100 nM was added, and finally KCl at increasing concentrations (0.25 to 50 mM). After 10 min incubation at RT, fluorescence changes were assessed (λ_{em}=580 nm, λ_{ex}= 540 nm).

Effect of inhibitors on V_m

We also evaluated the effect of various inhibitors on the V_m. These inhibitors were the ABC transporter blocker glibenclamide (GLIB; 100 μM); the classic Na^+, K^+-ATPase inhibitor ouabain (1 mM); the sodium ionophore monensin (1 μM); the chloride channel inhibitor 4,4′-di-isothiocyanate–2,2′-disulfonate sodium salt (DIDS; 500 μM); the glucose incorporation blocker phloretin (150 μM); the glucose transport inhibitor cytochalasin-B (Cytoc; 300 μM); the mitochondrial ATPase inhibitor oligomycin (20 μM); and the mitochondrial oxidative phosphorylation inhibitor carbonyl cyanide 4-trifluoromethoxyphenylhydrazone (FCCP; 1 μM).

The experiments were performed as explained before, but initially the cells were incubated at RT for 10 min and in the dark with the inhibitors and then 10 min with bisoxonol before measuring the fluorescence (λ_{em}=580 nm, λ_{ex}=540 nm).

Analysis of results

Each experiment evaluating glucose utilization was performed at least three times. All included positive and negative controls. Glucose concentrations (mM) were calculated according to instructions supplied by the company. The rate of glucose utilization was estimated by the disappearance of glucose from the medium. For calibration curve analysis (glucose 0 to 5 mM), the Microsoft Excel® 2007 and Prism Graph-Pad 5® programs were used.

Each experiment evaluating V_m was performed at least four times. All included positive and negative controls. To calculate membrane potential, we used the Nernst equation: $-V_m=-59.4 \log [K]_i/[K]_o$, where K_i is the intracellular potassium concentration (considered as 120 mM) (Vieira et al. 1995) and K_o is the KCl concentration added. A lineal correlation was established between bisoxonol fluorescence and each KCl concentration. For the Student *t* test analysis of the effect of inhibitors on V_m, the Microsoft Excel® 2007 and Prism Graph-Pad 5® programs were used.

Results

Glucose uptake in parasites isolated from DCL patients

The rate of glucose uptake by *Leishmania* is an indicator of cell viability, as it is closely associated with the metabolic status of the parasite (Seyfang and Duszenko 1991). Figure 1 illustrates the time course of glucose uptake in strains and

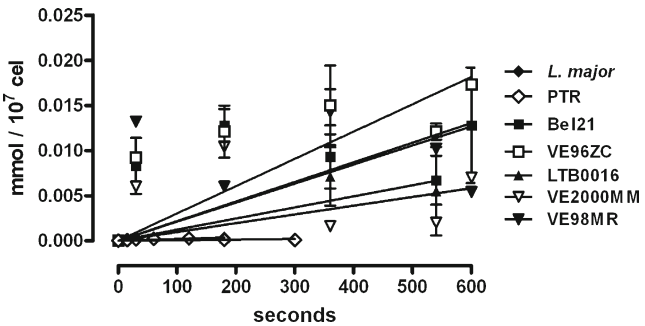

Fig. 1 The behavior of glucose uptake compared with time, in reference strains and in parasites isolated from DCL patients. The *slope* represents the rate of glucose uptake in each case

isolates herein analyzed. The data demonstrate the linear incorporation of glucose into the cells during the first 600 s. Strains and isolates accumulate the metabolite with different slopes. Glucose uptake spans over a large range of values, listed as rate of glucose uptake in Table 1. The data indicate that cells accumulate glucose in the following order: *L. mexicana* (VE96ZC > Bel21) = *L. amazonensis* (VE98MR) > *L. amazonensis* (LTB0016 = VE2000MM) ≫ *L. major* (*L. major* > PTR). These results suggest that: (1) *L. major*, responsible for cutaneous leishmaniasis (CL) in the Old World, accumulate glucose at a lower rate than *L. amazonensis* and *L. mexicana*, responsible for DCL in America; (2) in the pentamidine-resistant strain PTR, glucose accumulation is lower than in its *L. major* reference strain; and (3) in *L. amazonensis* and *L. mexicana* parasites isolated from DCL patients, glucose accumulation either increases or remains at similar values than in their corresponding reference strains.

Membrane potential (V_m) in parasites isolated from DCL patients

The membrane potential is the electromotive force needed for the incorporation of nutrients and solutes into cells. Interestingly, alterations in V_m have been associated with the physiology of chemoresistance in *Saccharomyces cerevisiae* (Cadek et al. 2004).

Table 2 summarizes V_m determined for each parasite tested. The data demonstrate that V_m spans from −170 to −200 mV for strains and isolates used herein. V_m of reference strains and isolates VE96ZC and VE98MR remain at values close to −200 mV. However, V_m significantly deviates toward depolarizing values in the isolate VE2000MM.

To further our analysis, we evaluated the response of isolates and reference strains to inhibitors that affect selective channels, conductances, or transporters. Figures 2, 3, and 4 illustrate the obtained results.

L. major and PTR, as well as LTB0016 and VE2000MM, V_m was not sensitive to DIDS, an inhibitor of anionic conductances; on the other hand, ouabain, the classical blocker of the Na^+, K^+-ATPase, decreased significantly the V_m of Bel21 and VE96ZC and the pentamidine-resistant *L. major* (PTR). Interestingly, the ABC transporter and K^+ channel blocker GLIB neither affected the V_m of any *L. amazonensis* analyzed herein nor that of Bel21, and inhibition of glucose transport either by phloretin or cytochalasin-B decreased significantly V_m in all cells tested. Finally, most cells were sensitive to oligomycin and FCCP; LTB0016 and VE98MR were sensitive to FCCP, and VE2000MM was insensitive to both inhibitors; *L. major* was sensitive to oligomycin. These results suggest that sensitivity of *Leishmania* membrane potential toward classical inhibitors of ion channels and transporters is species dependent and that strains isolated from patients suffering DCL but belonging to the same species differ in their behavior in comparison to their reference strains. That is: (1) the V_m of *L. mexicana* (VE96ZC) behaved similarly to that of Bel21, its reference strain, but additionally was sensitive to the ABC transporter and K^+ channel blocker GLIB, as well as to the hexose transporter inhibitor Phloretin; (2) the V_m of *L. amazonensis* (VE98MR) behaved similarly to that of LTB0016, its reference strain, but additionally was sensitive to the anionic current inhibitor DIDS; and (3) the V_m of *L. amazonensis* (VE2000MM) was sensitive only to glucose transporter inhibitors differing from VE98MR, a strain also belonging to *L. amazonensis* and isolated from a patient suffering DCL.

Discussion

In Venezuela, patients suffering DCL are treated with immunotherapy, either alone or in combination with Glucantime® or Miltefosine® (Zerpa and Convit 2009; Goto and Lindoso 2010). Relapses are common in DCL (Becker et al. 1999; Zerpa and Convit 2009; De Lima et al. 2010). Although this condition is usually associated with an anergic patient immune state (De Lima et al. 2010), treatment failure in DCL may also be associated with infection by chemoresistant parasites, overexpressing membrane transporters that decrease intracellular effective drug levels, or with epigenetic or pleiotropic physiological changes that sustain the resistant phenotype (Ponte-Sucre et al. 2013; Vanaerschot et al. 2011; Ouakad et al. 2011; Odiwuor et al. 2011).

The in vitro amastigote–macrophage model mimics the in vivo situation and correlates the clinical therapeutic response with reduced macrophage infection rate in the presence of increasing concentrations of drugs: Unfortunately it is a laborious and expensive method for monitoring resistance in

Table 1 Rate of glucose uptake in reference strains and in parasites isolated from DCL patients

L. major		*L. mexicana*		*L. amazonensis*		
*L. major**	PTR*	Bel21*	VE96ZC*	LTB0016**	VE2000MM	VE98MR**
mmol of glucose×10^{-5}/10^7 cell s^1						
0.19 ± 0.002	0.06 ± 0.004	2.12 ± 0.05	3.02 ± 0.04	1.23 ± 0.03	0.97 ± 0.5	2.17 ± 0.06

*p<0.001; **p<0.005 [1]mean +/- standard error of the mean

Table 2 Membrane potential (V_m) in reference strains and in parasites isolated from DCL patients

Species	V_m (mV) (95 % confidence interval)	Goodness of fit (R^2)
L. major	−180 (−196/−169)	0.99
L .major (PTR)	−200 (−219/−186)	0.96
L. mexicana (Bel21)	−200 (−220/−190)	0.84
VE96ZC	−195 (−240/−168)	0.54
L. amazonensis (LTB0016)	−212 (−230/−198)	0.89
VE2000MM	−170 (−190/−150)	0.73
VE98MR	−190 (−240/−170)	0.54

R^2 correlation coefficient

patient isolates. On the other hand, drug susceptibility of parasites isolated from patients is easy to achieve by reproducible in vitro methods. However, rapid isolation of parasites at diagnosis to prevent pathogen adaptation to culture is essential (Croft et al. 2006).

It is therefore imperative to describe markers easy to use in routine laboratory for guiding the anti-leishmania therapy. For example, previous results demonstrated that the level of calcein accumulation (an ABC transporter substrate) is a cellular marker of easy implementation in clinical laboratories; alternatively, fatty acid profiles in *Leishmania* isolates with natural resistance to nitric oxide and trivalent antimony have been claimed to be useful as biomarkers that could facilitate the identification of virulence and resistance mechanisms to particular chemotherapeutic agents. However until yet, they have a limited role in determining if treatment failure in DCL relies on infection by a chemoresistant parasite (Padrón-Nieves and Ponte-Sucre 2014; de Azevedo et al. 2014). Herein our aim has been to evaluate if relapses that occur in DCL patients might be associated with adaptation of the infecting parasites.

Cell homeostasis and degree of plasma membrane polarization (V_m) ensure the successful *Leishmania* adaptation and survival to the different environments where it lives through its life cycle. Herein we correlated parasite glucose accumulation with plasma V_m in reference strains and patient isolates. The results comprise a proof of concept that demonstrated that compared to reference strains, the tested isolates accumulated glucose at a higher rate, and yet their V_m remained unchanged. One isolate did not increase its glucose accumulation and interestingly expressed a depolarized V_m. These results indicate an efficient parasite capacity to accumulate glucose without major changes in their membrane potential, thus suggesting that in DCL patients, the infecting parasites—of either species—adapt to drug pressure increasing their fitness and adaptability. Increased fitness has also been described for

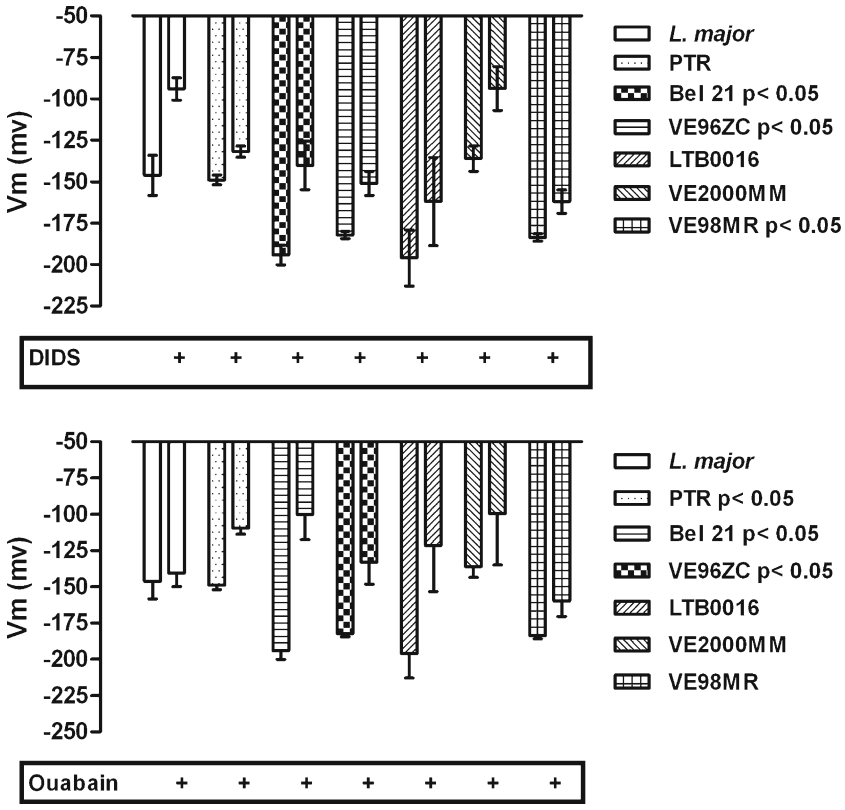

Fig. 2 Effect of DIDS and ouabain on the V_m in reference strains and in parasites isolated from DCL patients. $p<0.05$ in each strain or isolate V_m, comparing untreated vs. treated parasites

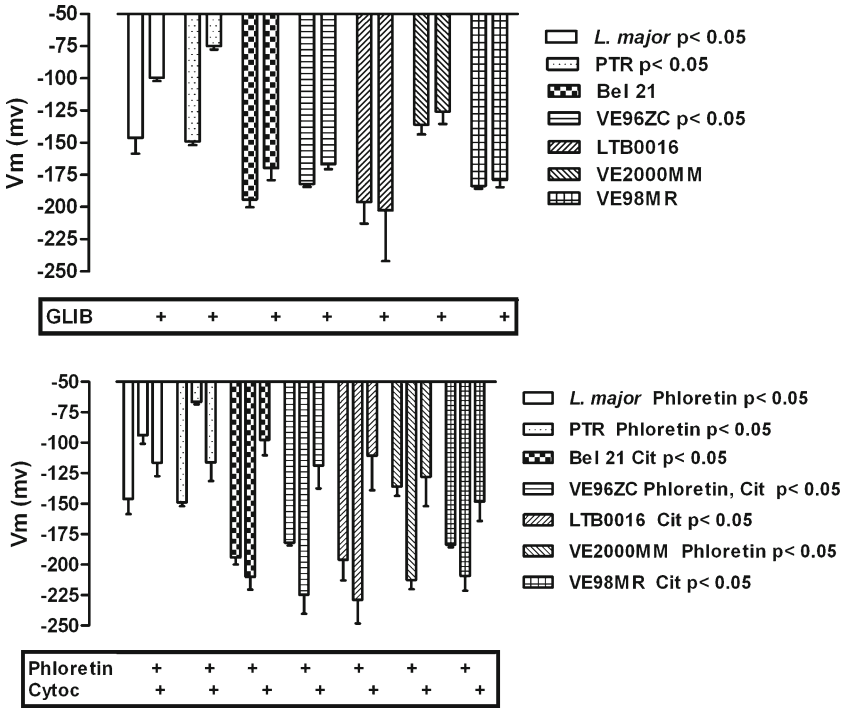

Fig. 3 Effect of GLIB, phloretin, and Cytoc on the V_m in reference strains and in parasites isolated from DCL patients. $p<0.05$ in each strain or isolate V_m, comparing untreated vs. treated parasites

antimonial-resistant *L. donovani* causing visceral leishmaniasis (Vanaerschot et al. 2010).

In previous studies, we have demonstrated that the experimental induction of chemoresistance in *L. mexicana* and *L. amazonensis* decreases the utilization of energy substrates (like glucose) in response to pressure imposed by the drug (Uzcategui et al. 2005; Machuca et al. 2006). Additionally, in the herein presented results, *L. major* PTR transfected with a pentamidine-associated chemoresistant gene also accumulates glucose at a lower rate than its reference strain. These results emphasize the special caution that should be taken since comparison of treatment outcome of parasites isolated from patients with in vitro results is rarely done.

Our results demonstrated that basal V_m remained at similar values in VE96ZC and VE98MR and their reference strains. On the contrary, V_m in VE2000MM depolarized, compared to its reference strain. A depolarized V_m implies a lower electromotive force. We evaluated the effect of some pharmacological inhibitors of ion channels and transporters on the determined membrane potential.

Ouabain, an inhibitor of the plasma membrane Na^+ K^+-ATPase fundamental for membrane polarization and maintenance of the electromotive force, decreased V_m of *L. mexicana* (Bel21 and VE96ZC) and *L. major* (PTR), but as has been previously demonstrated (Felibertt et al. 1998; de Almeida-Amaral et al. 2008), it did not collapse V_m of *L. amazonensis* strains or isolates. On the other hand, it has been suggested that chloride conductances play a secondary role in the generation and maintenance of V_m in *Leishmania* (Vieira et al. 1995). In our hands, DIDS, an inhibitor of chloride conductances and anion transporters, decreased V_m only in *L. mexicana*. Finally, the ABC transporter and K^+ channel blocker (GLIB) decreased V_m only in *L. mexicana* (VE96ZC). Altogether these results suggest that the role for Na^+ K^+-ATPase, chloride conductances, and K^+ channels in maintenance of *Leishmania* V_m is variable depending on the species

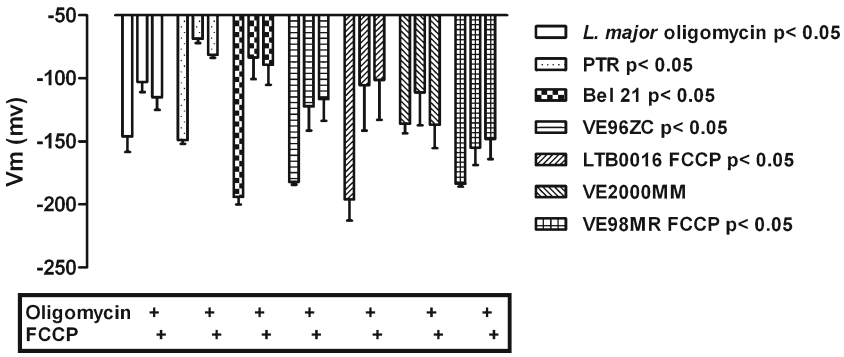

Fig. 4 Comparison of the effect of oligomycin (*second bar*) and FCCP (*third bar*) on the V_m in reference strains and in parasites isolated from DCL patients. $p<0.05$ in each strain or isolate V_m comparing untreated vs. treated parasites

and that *L. mexicana* is especially sensitive to these pharmacological blockers.

As the plasma membrane potential is dependent on cellular ATP production by both oxidative phosphorylation and glycolysis, the use of inhibitors to these functions should clarify the extent to which glycolytic and mitochondrial ATP production maintains the mitochondrial V_m (Kalbácová et al. 2003). Inhibition of glucose transport by phloretin, the nonspecific inhibitor of glucose transport, gave confusing results; it depolarized V_m in *L. major* strains, hyperpolarized V_m of VE96ZC (*L. mexicana*) and VE2000MM (*L. amazonensis*), and did not affect V_m in VE98MR (*L. amazonensis*) and reference strains. On the other hand, although the rate of glucose utilization by VE2000MM was similar to that of LTB0016, its homologous species, the V_m of VE2000MM but not LTB0016, was insensitive to the specific glucose transporter blocker cytochalasin-B. This result may imply that the isolate VE2000M express a glucose transporter that does not contain the conserved tryptophan residue present in helix XI, involved in cytochalasin binding, that has been demonstrated in *Babesia bovis* (Derbyshire et al. 2008).

L. mexicana (Bel21 and VE96ZC) V_m was sensitive to the mitochondrial inhibitors oligomycin and FCCP as has been previously demonstrated (Basselin and Robert-Gero 1998; Basselin et al. 2002), *L. amazonensis* (LTB0016 and VE98MR) to FCCP, *L. major* to oligomycin, and VE2000MM (*L. amazonensis*) was insensitive to any of these inhibitors. As in resistant parasites the collapse of mitochondrial V_m is a cell functional status indicator (Wilkes et al. 1997; Basselin et al. 2002) and redox systems control plasma membrane function in *Leishmania* (Bera et al. 2005), we might speculate that contrary to the other strains herein used, VE2000MM produces ATP mainly by means of glycolysis.

In conclusion, we postulate that in *L. mexicana* and *L. amazonensis*, parasites isolated from DCL patients, a strong modification of the parasite physiology might occur, adapted well to drug pressure, and increased their fitness; consequently, they had an efficient glucose uptake rate albeit not significant changes in membrane potential as their corresponding reference strains. This all means that they differ from their reference strains in their metabolic behavior. Metabolic changes have been previously demonstrated in antimonial-resistant *L. donovani* isolates (Berg et al. 2013). If our results reflect a similar situation still needs to be clarified. It is important to emphasize that increased infectivity and host manipulation skills have been described in *L. donovani* isolated from patients that suffered leishmaniasis and had treatment failure (Vanaerschot et al. 2013). However, there is as yet no description of whether these phenotypic changes are the result of mechanisms similar to the ones herein described for New World *Leishmania*.

What seems to be clear is that since the three patients suffer continuous relapses, the results presented herein constitute a proof of concept that indicates that the isolates adapted well to drug pressure in spite of the stress induced by the presence of drug and expressed an increased fitness. Further validation of the concepts established needs to be demonstrated.

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