Chapter 44

Cellular Markers for the Identification of Chemoresistant Isolates in *Leishmania*

Maritza Padrón-Nieves and Alicia Ponte-Sucre

Abstract

Markers to diagnose chemoresistance in infecting *Leishmania* parasites are urgently required. This is fundamental for patients who do not heal during or after treatment, as they are unresponsive, or patients who relapse at the end of the therapy, suffering from therapeutic failure. Glucose utilization is an indicator of cell viability that closely associates with metabolic activity. In *Leishmania*, glucose is a source of carbon atoms and is imported into the cell through specific transporters. In experimentally developed chemoresistant *Leishmania* parasites a significant decrease of the expression of glucose transporters as well as in the cellular accumulation glucose has been described. Alternatively, the electrical membrane potential is an essential parameter for the formation of the electromotive force needed for the acquisition of important nutrients and solutes (e.g., glucose) by cells, and changes in glucose concentration are suggested to constitute a physiological adaptation associated with a chemoresistant phenotype of *Leishmania* parasites. Here we describe easy methods to measure glucose uptake and the membrane potential in isolates from patient suffering leishmaniasis. Correlation between both parameters might be helpful to identify chemoresistant parasites. Results suggest that the measured kinetics of glucose utilization rate can be correlated with the plasma membrane potential and together used to differentiate between the performance of wild-type and reference parasites on the one hand and parasites isolated from patients with therapeutic failure on the other.

Key words Bis-oxonol, Drug resistance, Drug resistance markers, Glucose uptake, *Leishmania*, Plasma membrane potential, Therapeutic failure

1 Introduction

Chemotherapy research exploits qualitative and quantitative differences between host and pathogenic agents to design drugs that affect pathogen survival, while not affecting host integrity. The molecules eventually transform into human medicines that promote elimination of pathogenic agents [1]. However, therapeutic efficacy may not be adequate and consequently, the ghost of therapeutic failure (TF)—cases that do not heal during or after treatment, or do not respond from the beginning (unresponsiveness) and those which fail in their response after receiving the

Paul A. M. Michels et al. (eds.), *Trypanosomatids: Methods and Protocols*, Methods in Molecular Biology, vol. 2116, https://doi.org/10.1007/978-1-0716-0294-2_44,

corresponding therapy (relapses)—might emerge [2–4]. Among the multiple reasons that can support the existence of TF, that is, factors associated with the drug (presentation, route of administration, toxicity, pharmacokinetics, and cost) [5, 6], host (immunosuppression, comorbidity, noncompliance with the therapeutic regimen), or epidemiological conditions (risk of reinfection), only those associated with a pathogenic agent less susceptible to the drug—natural or acquired (under drug pressure)—favor the persistence of chemoresistant parasites [3].

Resistance implies that although a large percentage of pathogens living under drug pressure may die, selected parasites genetically different from "wild" cells survive. The selected cells express mutations that favor their persistence in the presence of the compounds and these mutations are transmitted to the offspring. The problems associated with this situation represent a public health challenge involving many diseases, including leishmaniasis. In fact, increases in the incidence of cases of leishmaniasis with TF and the need to identify which cases are related to parasite chemoresistance highlight the urgency of identifying easy-to-implement resistance markers, to be used in the laboratory routine for identifying infective parasites that express resistant phenotypes.

In the present chapter we describe how the correlation of results obtained by two methods could be implemented as easy-to-use resistance markers, that is, glucose uptake and plasma membrane potential.

Trophic activity is fundamental in the processes of transmission, development, adaptation, and survival of *Leishmania* [7]. Thus, the adaptive success of the parasite depends, to a large extent, on its ability to use substrates such as glucose [8]. In environments with high oxygen tension the parasites proliferate rapidly producing ATP through aerobic glycolysis, whereas when the oxygen level is low, they do so by anaerobic glycolysis [9]. The use of glucose has been shown to be restricted in media with low oxygen availability [10], as in the intracellular environment and in the intestine of the invertebrate vector, where the environment is hypoxic [7, 8, 11, 12].

Glucose uptake by the cell, independently of its subsequent metabolism [13, 14], offers a simple parameter to study the way how the cell's physiological state is affected. The determination of glucose is carried out in the supernatant of the culture at a determined time, for which the enzymatic method of Trinder [15] can be used. This reaction involves the oxidation of glucose in the presence of glucose oxidase to gluconic acid and hydrogen peroxide, and the formation of a red quinonimine, by reaction of the peroxide with 4-aminoantipyrine and phenol, in the presence of peroxidase. The amount of red quinonimine formed is proportional to the concentration of glucose in the sample and is determined spectrophotometrically (*see* Fig. 1). Specifically, we adapted the methodology originally described by Seyfang and Duszenko

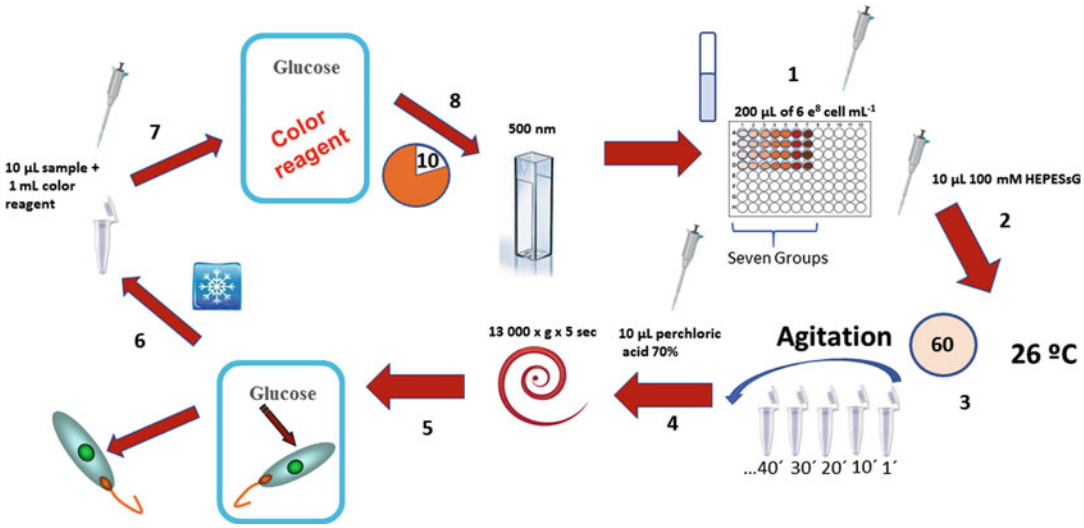

Fig. 1 Determination of glucose utilization rate

[16], to study glucose uptake in *Leishmania* parasites isolated from diffuse cutaneous leishmaniasis (DCL) patients with TF [17]. It may be a good cellular marker to differentiate chemosensitive and chemoresistant strains of *Leishmania*.

Differences in the electrical potential across membranes is a common characteristic both in prokaryotic and eukaryotic cells. These differences in plasma membrane potential ($\Delta\Psi p$) occur as a consequence of ionic concentration gradients (Na^+, K^+, H^+, Cl^-, ...) across membranes involving the action of different pumps [18–20]. In the case of *Leishmania* (*L.*) *donovani*, $\Delta\Psi p$ seems to be set by hydrogen and potassium ion diffusion gradients, while sodium does not seem to have a primary role [21]. Even more, as has been primarily demonstrated by Zilberstein and colleagues, *Leishmania* creates a protonmotive force across their plasma membrane that drives nutrient transport [22, 23].

At rest, mammalian cells have a $\Delta\Psi p$ in the range of 40–90 mV (~70 mV), the interior being negative with respect to the exterior [20]. By convention, the negative interior $\Delta\Psi p$ is expressed with a minus sign (−40 to −90 mV). In protists such as *Leishmania*, the $\Delta\Psi p$ depends on the energy metabolism and the life cycle of the parasite, varying between −75 mV in amastigotes [22–24] and −113 mV in promastigotes [25]. Hyperpolarization occurs as an increase in the magnitude of the $\Delta\Psi p$ at rest and it is expressed with a lower value than the previous one, while depolarization represents the opposite situation.

The $\Delta\Psi p$ is fundamental for the formation of the protonmotive force necessary for nutrient and other important solute acquisition by the parasites, as well as for permitting the interaction with the

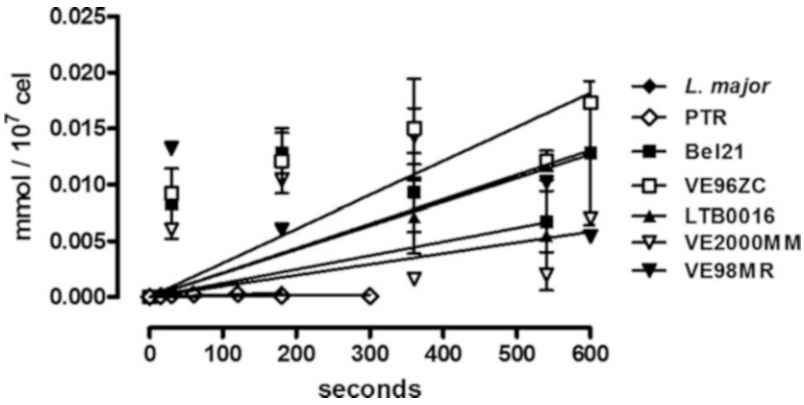

Fig. 2 Comparison of the rate of glucose uptake by reference strains and parasites isolated from DCL patients (Reprinted by permission from Nature/Springer/Palgrave, Parasitology Research 2014; 113(6):2121–2128)

host [18, 19, 22, 24–26]. Thus, the $\Delta\Psi p$ may reflect the state of energy metabolism, cellular homeostasis, and the physical integrity of the plasma membrane.

Some fluorescent markers, such as the lipophilic anion bis-oxonol [bis-(1,3-diethylthiobarbituric acid) trimethinaoxonol], can be used to indirectly measure $\Delta\Psi p$ in *Leishmania* (*see* Fig. 2) [27, 28]. In the presence of cellular depolarization, bis-oxonol undergoes a conformational change, increasing its emission of light by fluorescence. When the cells repolarize, the anion dissociates from the membrane and decreases the emission [27, 28]. To measure the $\Delta\Psi p$ of *Leishmania*, we use the bis-oxonol fluorescent compound according to the methodology initially described by Vieira et al. [25], with modifications. This technique has been used to study the leishmanicidal effect of peptide antibiotics on promastigotes of *Leishmania* (*L.*) *donovani* [29] and $\Delta\Psi p$ in New World *Leishmania* parasites isolated from DCL patients with TF [17] and may be a good cellular marker to differentiate chemosensitive and chemoresistant strains of *Leishmania*. To measure the $\Delta\Psi p$, it is mandatory to perform some calibration procedures to determine the optimal cell density to be used, as well as the extracellular and intracellular bis-oxonol concentration.

For the herein described methods, the parasites isolated from lesions (isolates) were obtained from three patients that regularly attended the Dermatology Department of the Institute of Biomedicine, MPPS-UCV-Caracas-Venezuela. The patients suffered DCL with TF to treatment with meglumine antimonate. The isolates were identified by molecular biology [30] as belonging to the *Leishmania* (*L.*) *mexicana* and *Leishmania* (*L.*) *amazonensis* groups and were kept frozen in liquid nitrogen until their use to minimize changes in their phenotype. Further details are given in the Methods section.

2 Materials

All solutions should be prepared either with double distilled water or deionized water (impedance of 18 MΩ cm at 25 °C) and analytical grade reagents. Each method describes the storage characteristics of the solutions to be used. Follow strictly the instructions and be aware to dispose of in an appropriate way the waste, following suitable regulations.

2.1 Solutions

1. Biphasic medium of blood agar (modified NNN medium) prepared according to standard procedures. Solid phase: base agar with 15% defibrinated rabbit blood and sodium penicillin (100 U/mL). Liquid phase: glucose solution 1.5% w/v, NaCl 0.85% w/v, male urine 5% v/v, and bovine fetal serum (inactivated at 56 °C for 30 min) 10% v/v (*see* **Note 1**).
2. Saline phosphate buffer solution (PBS): 136 mM NaCl, 2.6 mM KCl, 10 mM $Na_2HPO_4 \cdot 2H_2O$, 1.7 mM KH_2PO_4 (*see* **Note 1**).
3. HEPES salts (HEPESs): 10 mM HEPES, 132 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.3 (*see* **Note 2**).
4. HEPES salts glucose (HEPESsG): 10 mM HEPES, 132 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM glucose, pH 7.3 (*see* **Note 2**).
5. Perchloric acid 70% (70-PA) (*see* **Note 3**).
6. *N*-methylglucamine (NMGCl): 10 mM HEPES, 140 mM NMGCl, 0.5 mM $MgCl_2$, 11 mM glucose, pH 7.3 (*see* **Note 2**).
7. Bis-oxonol [bis-(1,3-diethylthiobarbituric acid)trimethinaoxonol] (Molecular Probes) [28] 10 μM (stock solution dissolved in DMSO) (*see* **Note 4**).
8. Valinomycin 100 μM (stock solution in DMSO) (*see* **Notes 4** and **10**).
9. KCl 4 mM (stock solution in water).

2.2 Parasites

2.2.1 Reference Species Certified by the World Health Organization (WHO)

1. *L. amazonensis* (MHOM/BR/77/LTB0016).
2. *Leishmania* (*L.*) *major* (MHOM/IL/81).
3. *L. major* (MHOM/SU/73/5ASKH).
4. *L. mexicana* (MHOM/BR/82/Bel21).
5. *L. major PTR* (MHOM/IL/80/Friedlin/PTR, a cosmid transfected strain containing PRP1, an ABC transporter conferring pentamidine resistance in *L. major* [31].

2.2.2 Parasites Isolated from Patient Lesions

6. *L. mexicana*—MHOM/VE/ 1996/ZC-(VE96ZC).
7. *L. amazonensis*-MHOM/VE/1998/MR-(VE98MR).
8. *L. amazonensis*-MHOM/VE/2000/MM-(VE2000MM).

Parasites of species 1 and 2 were donated by Dr. Lionel Schnur, The Hebrew University-Hadassah Medical School, Jerusalem; species 3, 4, 6, 7, and 8 by Dr. Noris Rodríguez, Instituto de Biomedicina, Universidad Central de Venezuela, Caracas, Venezuela; and species 5 by Dr. Paulo Cotrim Dpto. Moléstias Infecciosas e Parasitárias, Instituto de Medicina Tropical de São Paulo, São Paulo, Brazil.

3 Methods

3.1 Parasite Culture and Estimation of Cell Density

1. Parasites are thawed according to conventional protocols and subcultured weekly in NNN semisolid blood agar, at 26 °C, with glucose solution. The parasites are seeded at 5×10^5 cells/mL and cultivated until growth reaches the initial stationary phase, just before the plateau.
2. Dilute an aliquot, 10 μL of a culture suspension with 0.04% trypan blue in PBS and place it under a microscope (40× magnification) using a Neubauer chamber. Count live (colorless) parasites. Calculate cell density using the following formula according to the manufacturer's recommendation: $N°$ of cells/mL = $N°$ of cells $\times 10^4 \times$ dilution factor.

3.2 Glucose Utilization Rate

Glucose uptake is determined in the exponential phase of parasite growth by measuring the import of the substrate into the cell independently of its subsequent metabolism [13, 14]. Specifically, glucose disappearance from the medium with time is measured by an adaptation of a methodology previously described [15]. The identity of the transporters involved has been demonstrated previously [32]. Figure 1 illustrates the glucose uptake method herein used step by step as follows:

1. After centrifugation (500 × *g* for 10 min) and washing promastigotes three times with PBS, resuspend the cells (gently, using Pasteur pipettes) in 200 μL of HEPESs, at a density of 2×10^8 cells/mL.
2. Place 200 μL of 2×10^8 cells/mL resuspended in HEPESs in a 96 well plate. Make seven groups of quadruplicates.
3. Add 10 μL of HEPESsG to obtain a final concentration of 5 mM glucose (*see* **Note 5**).
4. Incubate at 26 °C with agitation. Withdraw the first group of tubes from the bath at min 1 and then the remaining groups every 10 min until completion at 1 h.

5. To the withdrawn tubes add 10 μL of 70-PA to stop the reaction (*see* **Note 3**).
6. Centrifuge for 5 s at 13,000 × *g* at room temperature (RT).
7. Store the supernatant at 4 °C until glucose determination.
8. To 10 μL of each sample, including the reference glucose standard, add 1 mL of the Cromatest® Kit color reagent.
9. Incubate for 10 min at RT, measure the absorbance at 500 nm to determine the glucose concentration (*see* **Note 6**).

Perform each experiment determining glucose utilization at least three times in quadruplicate. Include blank and controls: i.e. one tube with 5 mM glucose concentration as standard; one tube including only the color reagent and one tube including only HEPESsG.

The rate of glucose utilization is estimated from the decrease of glucose levels in the medium. For curve analysis, we use the Microsoft Excel® 2007 and Prism Graph-Pad 5® programs.

Figure 2 depicts an example of the time course of glucose uptake by strains and isolates. The slope of each curve represents the rate of glucose uptake for each case. In all cases and for all strains and isolates a linear incorporation of glucose into the cells during the first 600 s was demonstrated. The slopes obtained for each reference strain or isolate, expressed as mmol of glucose × 10^{-5}/10^{7} cells sec, and 95% confidence intervals, are summarized in Table 1. The data represented in this table compare the rates of glucose import for each isolate and its corresponding reference strain. The results indicate that cells accumulate glucose following the order *L. mexicana* (VE96ZC > Bel21) = *L. amazonensis* (VE98MR) > *L. amazonensis* (LTB0016 = VE2000MM) ≫ *L. major* (*L. major* > PTR).

3.3 Plasma Membrane Potential ($\Delta\Psi$p)

In eukaryotic cells, membrane potentials occur in several organelles, including mitochondria. The membrane potential probe bis-oxonol only fluoresces in the microenvironment of a membrane, and enters and exits the cell in response to the charge on the plasma membrane. It can enter depolarized cells and bind to

Table 1
Rate of glucose utilization by reference strains and patient isolates

mmol of glucose × 10^{-5}/10^{7} cells second (95% confidence intervals)						
L. major		***L. mexicana***		***L. amazonensis***		
*L. major**	PTR*	Bel21**	VE96ZC**	LTB0016***	VE2000MM	VE98MR***
0.19 + 0.002 (0.11–0.27)	0.06 + 0.004 (0.00–0.015)	2.12 + 0.05 (0.98–3.25)	3.02 + 0.04 (2.00–3.90)	1.23 + 0.03 (0.3–2.13)	0.97 + 0.5 (0.00–0.22)	2.17 + 0.06 (0.53–3.81)

*' **$p < 0.001$, ***$p < 0.005$

intracellular proteins or membranes, exhibiting an enhanced (brighter) fluorescence. As the inner leaflet becomes more negative (hyperpolarized), bis-oxonol leaves the cell and its signal decreases [33]. Although bis-oxonol should be largely excluded from mitochondria because of their overall negative charge, the signal produced by this dye provides a ratio that includes these (and other) organelles.

Before performing the experimental protocol, calibrations should be accomplished: that is, the conditions for linearity of fluorescence *vs.* cell density and the optimal extracellular and intracellular concentration of bis-oxonol. This allows the linear relationship with the fluorescence to be determined.

3.3.1 Calibration of Cell Density vs. Fluorescence (ΔF)

1. Place cells at variable densities (from 10^5 to 10^7 in 200 μL of HEPESsG) in a 96-well plate.
2. Add bis-oxonol (2 μL of 10 μM stock solution) to reach a concentration of 0.1 μM.
3. Incubate at RT for 10 min in darkness. Record the fluorescence changes (540 nm (exc); 580 nm (emi)) for 60 s.
4. Establish linearity between cell density and fluorescence changes (*see* **Note 7**).

3.3.2 Calibration of Extracellular and Intracellular Concentration of Bis-Oxonol vs. Δf

1. Place cells at the optimal density in 200 μL of HEPESsG in a 96-well plate.
2. Add bis-oxonol, [varying volumes (1–10 μL) stock solution] to reach concentrations (0.05–0.5 μM).

Skip **steps 3** and **4** from Fig. 3.

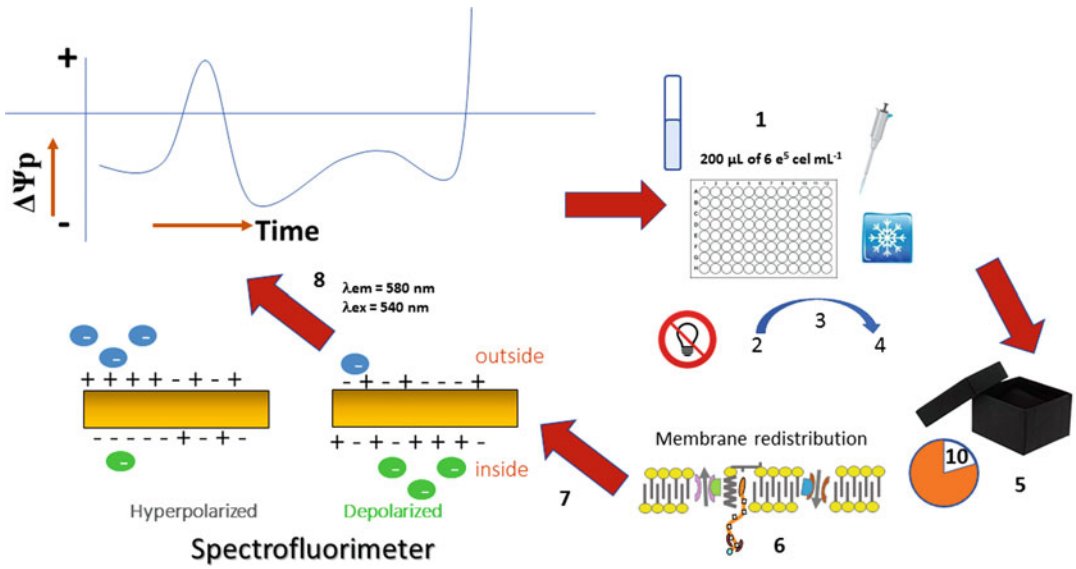

Fig. 3 Determination of $\Delta\Psi p$

5. Incubate at RT for 10 min.
6. Record the fluorescence changes (540 nm (exc)/580 nm (emi)) for 60 s (*see* **Note 7**).
7. Establish the linearity between bis-oxonol concentration and fluorescence changes (*see* **Notes 8** and **9**).

3.3.3 Experimental Protocol for the Evaluation of ΔΨp

Figure 3 illustrates the method herein used to determine $\Delta\Psi p$ step by step as follows:

1. Place cells at the optimal density as determined from the calibration curve (described in Subheading 3.3.2) in 200 μL NMGCl solution in a 96-well plate (*see* **Notes 8** and **9**).
2. Add valinomycin (2 μL of 100 μM stock solution) to reach a concentration of 1 μM (*see* **Notes 3** and **10–12**) [34].
3. Add bis-oxonol (2 μL of 10 μM stock solution) to reach a concentration of 0.1 μM.
4. Add KCl. From the original stock perform dilutions in water to prepare KCl solutions of 25–3000 μM. Add 2 μL of each dilution to successive wells to obtain varying KCl concentrations from 0.25 to 20 mM (*see* **Note 13**).
5. Incubate at RT in the darkness for 10 min.
6. Record the fluorescence changes (540 nm (exc); 580 nm (emi)) for 60 s.
7. Establish the linearity between fluorescence and estimated plasma membrane potential $\Delta\Psi p$ according to the extracellular KCl concentration.
8. Determine the basal $\Delta\Psi p$ and its depolarizing behavior in increasing concentrations of KCl for each isolate, to later compare with the reference species (*see* **Note 14**).

Perform each experiment to determine the $\Delta\Psi p$ (please follow instructions as depicted in Fig. 3) at least four times in quadruplicate, in each case with positive and negative controls included.

Figure 4 and Table 2 present experimental results obtained using these protocols for our reference strains and parasites isolated from DCL patients. First, a linear correlation was established between bis-oxonol fluorescence and KCl concentration. For the Student *t* test analysis of the effect of inhibitors on $\Delta\Psi p$, the Microsoft Excel® 2007 and Prism Graph-Pad 5® programs were used. The calibration curves of plasma membrane potential for each strain are depicted in Fig. 4, displaying the linear correlation between fluorescence (540 nm/580 nm) and plasma membrane potential. Additionally, Table 2 summarizes the $\Delta\Psi p$ determined from the corresponding slopes for each strain tested. The determined data demonstrate that $\Delta\Psi p$ spans from −170 to −200 mV

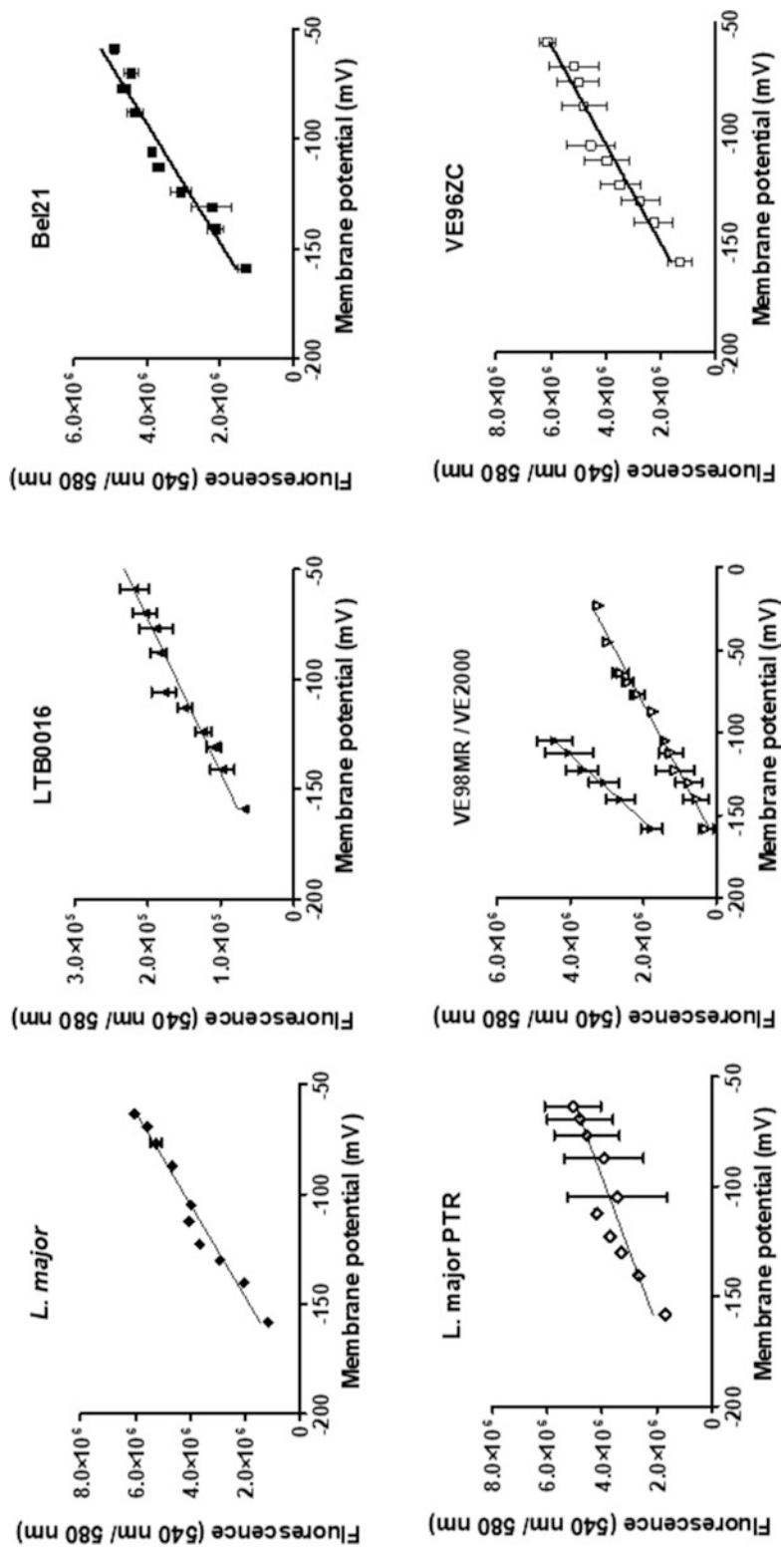

Fig. 4 The linear relationship between plasma membrane potential and measured fluorescence in reference strains and parasites isolated from DCL patients

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

Species	Vm [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 (Bel 21)	−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

for strains and isolates used herein. $\Delta\Psi p$ of reference strains and isolates VE96ZC and VE98MR remain at values close to −200 mV. However, $\Delta\Psi p$ significantly deviates toward depolarizing values in isolate VE2000MM.

3.4 Conclusion

Glucose utilization by parasites is an indicator of cell viability, as it is closely associated with metabolic activity. On the other hand, the plasma membrane potential is an essential parameter in the formation of the protonmotive force needed for the acquisition of important nutrients and solutes (e.g., glucose) in many organisms and cells, including *Leishmania*. Results obtained by the herein described methods 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*, both 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 as in their corresponding reference strains. Additionally, as the plasma membrane potential is the force needed for import of nutrients and other solutes into cells, results suggest that parasites accumulate glucose without a significant modification of the $\Delta\Psi p$, a result which could constitute a physiological change that may reflect an adaptation of the parasite against chemotherapeutic agents [35], initially defined as "fitness" [36]. As these results thus suggest that chemoresistance may involve a physiological response to the drug pressure—a metabolic adaptation—that modulates the use of energy substrates, the herein described and standardized methods for evaluating both parameters and their correlation, could be helpful in the identification of chemoresistant infecting parasites. It is interesting to note that previously we have used these two methods together to identify which ions are

responsible for the plasma membrane potential in *Leishmania* parasites, and if these ions differ in chemosensitive and chemoresistant parasites [17], as well as to analyze the mechanism of action of natural products and semisynthetic molecules derived from them and designed as potential antileishmania products (Alcazar, personal communication), and the mechanisms by which neuropeptides may exert their action as chemotactic agents (Giammarressi, personal communication). Thus, the ease of their implementation makes them treasured for laboratory routine as well as for the molecular laboratory.

4 Notes

1. Store at 4 °C.
2. The osmolarity of this solution should be checked to have the adequate physiological value of 298 mOsm/L.
3. Perchloric acid is strongly oxidizing. Thus, when you work with it, please use fume hoods with a wash-down capability to prevent accumulation of oxidizers in the ductwork.
4. DMSO is a liquid that should be handled with precaution. If working in cold climates remember that pure DMSO has a melting point of 18.5 °C (64 °F) and if frozen DMSO must be thawed before it can be used. To preserve the purity of the solution it is wise to transfer an aliquot to a small tube and use it rather than the big flask, since it very easily captures water. Please use fume hoods with a wash-down capability to prevent accumulation of DMSO gases.
5. Calibration curves should be performed in the range of 0–5 mM glucose.
6. Glucose concentrations (mM) should be calculated according to instructions supplied by the company. Glucose concentration = 5.5 × sample OD/standard OD = mmol/L.
7. It is recommended to make a minimum of four replicates of each condition.
8. To establish the linearity between intracellular bis-oxonol concentration and fluorescence, the cells must be heated for 15 min at 60 °C to collapse the plasma membrane potential; under these conditions bis-oxonol freely distributes in the cell so that both fluorophore concentrations [internal and external] are similar.
9. These cells must be resuspended in the NMGCl solution where all cations are replaced by NMGC1 and therefore cells are dissolved in monovalent cation-free solution.

10. Since this compound increases the fluorescence background, you should be very careful when using it; background values must be subtracted from the values of fluorescence.
11. Valinomycin is a selective dodecadepsipeptide antibiotic ionophore specific for potassium [34].
12. These cells must be resuspended in the HEPESs.
13. KCl working solutions.

Sol.	[KCl] (μM)	Solute (μL)	Solvent distilled H_2O (μL)	Total (μL)	(2 μL in 200 μL) (1:100) (mM)
0	*4000*	*KCl 0.5964 g (MW 74.56 g/mol)*	*2000*	*2000*	–
12	3000	375 Sol. 0	125	500	30
11	2000	250 Sol. 0	250	500	20
10	1000	125 Sol. 0	375	500	10
9	800	100 Sol. 0	400	500	8
8	600	75 Sol. 0	425	500	6
7	400	50 Sol. 0	450	500	4
6	*200*	*100 Sol. 0*	*1900*	*2000*	*2*
5	150	375 Sol. 6	125	500	1.5
4	100	250 Sol. 6	250	500	1
3	75	187.5 Sol. 6	312.5	500	0.75
2	50	125 Sol. 6	375	500	0.5
1	25	62.5 Sol. 6	437.5	500	0.25

14. The plasma membrane potential ($\Delta\Psi p$) is calculated according to the KCl concentration added to the wells using the Nernst equation: $\Delta\Psi p = -59.16 \log([K^+]i/[K^+]o)$, where $[K^+]i$ is the intracellular potassium concentration (considered as 120 mM) [22] and $[K^+]o$ is the KCl concentration added. A linear correlation is established between bis-oxonol fluorescence and KCl concentration.

Acknowledgments

The authors are grateful to Dr. Emilia Diaz, Med. Vet. Wilmer Alcazar, and Med. Doc. Michelle Giammarressi for details related to the methods herein described and to Mrs. Pilar Rodríguez for her technical assistance. Likewise, they are grateful for the support conferred by the Alexander von Humboldt Foundation and the

Siebold-Collegium Institute for Advanced Studies, University of Würzburg, Germany, to Alicia Ponte-Sucre. The authors are grateful to the Universidad Central de Venezuela Council for Research, grants CDCH-UCV PI-09-8717-2013/1 and PG-09-8646-2013/1. This project was approved by the Ethical Committee of the Institute of Biomedicine, Universidad Central de Venezuela.

References

1. Barret M Bolelart M, Castillo-Riquelme M et al. (2010) Research priorities for Chagas disease, human African *Trypanosomiasis* and *Leishmaniasis*. Technical report of the TDR Disease Reference Group on Chagas disease, Human African Trypanosomiasis and Leishmaniasis
2. Salaam-Blyther T. (2013) Neglected tropical diseases: background, responses, and issues. R41607 CRS report for Congress Congressional Research Service. www.crs.gov
3. G-Science Academies Statements (2013) Drug resistance in infectious agents – a global threat to humanity innovation. www.who.int/pmnch/media/membernews/2011/20110407_who_whd/en/
4. Ponte-Sucre A, Gamarro F, Dujardin JC et al (2017) Drug resistance and treatment failure in leishmaniasis: a 21st century challenge. PLoS Negl Trop Dis 11(12):e0006052. https://doi.org/10.1371/journal.pntd.0006052
5. Reveiz L, Maia-Elkhoury ANS, Nicholls RS et al (2013) Interventions for american cutaneous and mucocutaneous leishmaniasis: a systematic review update. PLoS One 8:1–14
6. Croft SL, Sundar S, Fairlamb AH (2006) Drug resistance in leishmaniasis. Clin Microbiol Rev 19:111–126
7. Durán C, Quiroga MF, Díaz-bello Z et al (2009) *Leishmania chagasi* y *Trypanosoma cruzi*: conducta trófica en cultivos axénicos puros y mixtos. Bol Malariol y Salud Ambient 49:97–106
8. Subramanian A, Jhawar J, Sarkar RR (2015) Dissecting *Leishmania infantum* energy metabolism – a systems perspective. PLoS One 10:e0137976. (1–34)
9. Michels PAM, Bringaud F, Herman M et al (2006) Metabolic functions of glycosomes in trypanosomatids. Biochim Biophys Acta 1763:1463–1477
10. Furuya T, Kessler P, Jardim A et al (2002) Glucose is toxic to glycosome-deficient trypanosomes. Proc Natl Acad Sci U S A 99:14177–14182
11. Saunders EC, Ng WW, Kloehn J et al (2014) Induction of a stringent metabolic response in intracellular stages of *Leishmania mexicana* leads to increased dependence on mitochondrial metabolism. PLoS Pathog 10:e1003888. (1–15)
12. Burchmore RJS, Hart DT (1995) Glucose transport in amastigotes and promastigotes of *Leishmania mexicana mexicana*. Mol Biochem Parasitol 74:77–86
13. Seyfang A, Landfear SM (1999) Substrate depletion upregulates uptake of myo-inositol, glucose and adenosine in *Leishmania*. Mol Biochem Parasitol 104:121–130
14. Machuca C, Rodríguez A, Herrera M et al (2006) *Leishmania amazonensis*: metabolic adaptations induced by resistance to an ABC transporter blocker. Exp Parasitol 114(1):1–9
15. Trinder P (1969) Determination of glucose in blood using glucose oxidase with an alternative oxygen aceptor. Ann Clin Biochem 6:24–27
16. Seyfang A, Duszenko M (1991) Specificity of glucose transport in *Trypanosoma brucei*. Effective inhibition by phloretin and cytochalasin B. Eur J Biochem 202:191–196
17. Padrón-Nieves M, Diaz E, Machuca C et al (2014) Correlation between glucose uptake and membrane potential in *Leishmania* parasites isolated from DCL-patients with therapeutic failure: a proof of concept. Parasitol Res 113(6):2121–2128
18. Konings W, Albers S-V, Koning S et al (2002) The cell membrane plays a crucial role in survival of bacteria and archaea in extreme environments. Antonie Van Leeuwenhoek 81:61–72
19. Nolan DP, Voorheis HP (2000) Factors that determine the plasma-membrane potential in bloodstream forms of *Trypanosoma brucei*. Eur J Biochem 267:4615–4623
20. Purves D, Augustine GJ, Fitzpatrick D et al (2001) Electrical signals of nerve cells. In: Purves D, Augustine GJ, Fitzpatrick D et al (eds) Neuroscience. Sinauer Associates, Sunderland, MA, pp 31–46

21. Glaser TA, Utz GL, Mukkada AJ (1992) The plasma membrane electrical gradient (membrane potential) in *Leishmania donovani* promastigotes and amastigotes. Mol Biochem Parasitol 51:9–15
22. Zilberstein D, Dwyer DM (1985) Protonmotive force-driven active transport of D-glucose and L-proline in the protozoan parasite *Leishmania donovani*. Proc Natl Acad Sci U S A 82:1716–1720
23. Zilberstein D, Dwyer DM (1988) Identification of a surface membrane proton-translocating ATPase in promastigotes of the parasitic protozoan *Leishmania donovani*. Biochem J 256:13–21
24. Marchesini N, Docampo R (2002) A plasma membrane P-type H^+-ATPase regulates intracellular pH in *Leishmania mexicana amazonensis*. Mol Biochem Parasitol 119:225–236
25. Vieira L, Slotki I, Cabantchik ZI (1995) Chloride conductive pathways which support electrogenic H+ pumping by *Leishmania major* promastigotes. J Biol Chem 270:5299–5304
26. Souto-Padron T (2002) The surface charge of trypanosomatids. An Acad Bras Cienc 74:649–675
27. Bene L (2013) Oxonol has the potential to probe membrane fields. Cytometry A 83:608–611
28. DiSBAC2(3) (bis-(1,3-diethylthiobarbituric acid)trimethine oxonol). https://www.thermofisher.com/order/catalog/product/B413?SID=srch-srp-
29. Vanaerschot M, Maes I, Ouakad M et al (2010) Linking in vitro and in vivo survival of clinical *Leishmania donovani* strains. PLoS One 8(8): e12211. https://doi.org/10.1371/journal.pone.00122
30. Rodríguez N, De Lima H, Aguilar CM et al (2002) Molecular epidemiology of cutaneous leishmaniasis in Venezuela. Trans R Soc Trop Med Hyg 96(S1):105–109
31. Coelho AC, Beverley SM, Cotrim PC (2003) Functional genetic identification of PRP1, an ABC transporter superfamily member conferring pentamidine resistance in *Leishmania major*. Mol Biochem Parasitol 130:83–90
32. Uzcategui NL, Figarella K, Camacho N et al (2005) Substrate preferences and glucose uptake in glibenclamide-resistant *Leishmania* parasites. Comp Biochem Physiol C Toxicol Pharmacol 140(3–4):395–402
33. Adams DS, Levin M (2012) Measuring resting membrane potential using the fluorescent voltage reporters DiBAC4(3) and CC2-DMPE. Cold Spring Harb Protoc 4:459–464. https://doi.org/10.1101/pdb.prot067702.
34. Díaz-Achirica P, Ubach J, Guinea A et al (1998) The plasma membrane of *Leishmania donovani* promastigotes is the main target for CA(1-8)M(1-18), a synthetic cecropin A-melittin hybrid peptide. Biochem J 330:453–460
35. Padrón-Nieves M, Machuca C, Díaz E et al (2014) Correlation between glucose uptake and membrane potential in *Leishmania* parasites isolated from DCL patients with therapeutic failure: a proof of concept. Parasitol Res 113:2121–2128
36. Natera S, Machuca C, Padrón-Nieves M et al (2007) *Leishmania* spp.: proficiency of drug resistant parasites. Int J Antimicrob Agents 29:637–642