Sensitivity of *Leishmania* spp. to Glibenclamide and 4-Aminopiridine: A Tool for the Study of Drug Resistance Development

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We have demonstrated that Leishmania *spp. grown as promastigotes, are sensitive to the K^+ channel inhibitors 4-aminopyridine and glibenclamide. Their host cells, the macrophages, are not affected by similar concentrations of the drugs. We have also initiated the molecular characterization of the mechanisms involved in the development of drug resistance to glibenclamide by the parasite. Therefore, we have selected experimentally and begun to characterize the Venezuelan* Leishmania *(*Leishmania*) strain, NR resistant to glibenclamide [NR(Gr)]. The analysis of genomic DNA evidenced the existence of a fragment which apparently is amplified in NR(Gr). The fragment recognized by the pgpA probe, related to the* Leishmania *P-glycoprotein family and which was originally isolated from* L. tarentolae, *showed a size polymorfism between the sensitive and the resistant strain. These results suggest that the development of resistance to glibenclamide in the strain NR(Gr) might be associated with the amplification of the ltpgpA or related gene(s).*

Key words: *Leishmania* - chemotherapy - glibenclamide - 4-aminopyridine - drug resistance - P-glycoprotein

Leishmaniasis is a disease with different clinical manifestations. The toxicity of conventional drugs such as the antimonials, and the possible development of resistance by the parasite to such compounds, emphasize the importance of the search for alternative strategies, both for its prevention and its treatment (Olliaro & Bryceson 1993). The accurate and early diagnoses of the disease, and its effective control, are extremely important and must be adequately addressed.

From the chemotherapeutic point of view, the development and evaluation of new drugs is mainly based on their efficacy against parasites. Such activity is sometimes opposed either by the complex life cycle of the parasite or by the intracellular nature of the developmental stages of the organisms as happens in *Leishmania* (Olliaro & Bryceson 1993). In this work we have attempted to evaluate the effect of 4-aminopyridine and glibenclamide, two classical inhibitors of K^+ channels, on the viability of *Leishmania* promastigotes and analyze some of the molecular mechanisms involved in the development of resistance to glibenclamide.

MATERIALS AND METHODS

Drugs - The drugs used were the classic K^+ channel inhibitors 4-aminopyridine (4-AP) for voltage gated channels, and glibenclamide (GLIB) for ATP gated channels. 4-AP was dissolved in deionized water to 0.5 M and GLIB was dissolved in ethanol to 4×10^{-3} M. Both drugs were further diluted in Schneider's *Drosophila* medium to the desired concentrations. As GLIB has an afinity for serum albumin greater than 96%, a correction has to be done to estimate the free drug concentration in the medium. Such estimation was done using the methodology described by Bennet et al. (1996). 4-AP does not have a high affinity for serum proteins as albumin thus, the free drug concentration in the medium is considered to be the same added.

Strains, cell lines and culture conditions - Table shows the different *Leishmania* strains used in this study. The Venezuelan strain NR was originally

isolated in 1980 from a Venezuelan patient with tegumentary diffuse leishmaniasis and was kindly provided by Dr Angel Hernández, Instituto de Biología Celular, Universidad Central de Venezuela. Its identification was carried out in this study by RFLP analysis of the ß-tubulin gene region as previously described (Mendoza-León et al. 1995).

The leishmanicidal activity of the compounds was studied in *Leishmania* NR promastigotes cultured in Schneider's *Drosophila* medium (GIBCO) supplemented with 10% fetal calf serum. The parasites were harvested at their stationary-phase of growth, the cell suspension was adjusted to a density of 2×10^6 cells ml^{-1} in a final volume of 0.1 ml of fresh medium. Aliquots of stationary-phase *Leishmania* were then exposed to various concentrations of the drug (i.e., 10^{-10}-10^{-2} M) at room temperature for 2 or 4 hr. Subsequently, the number of live cells was estimated in each experimental vial and compared to the number of live cells of control vials incubated without drugs. The viability of *Leishmania* was determined by a modification of a previously described procedure (Jackson et al. 1985). At the end of the incubation time, the cells were washed twice in phosphate buffer (PBS), resuspended in the same buffer at the same density, stained for 2 min at 25°C with 125 μg ml^{-1} fluorescein diacetate and observed with a Nikon fluorescence microscope at 495 nm. The results are expressed as the percentage of live parasites at each drug concentration.

The isolation of a resistant *Leishmania* strain was assayed in three isolates of New World *Leishmania*, i.e., the Venezuelan strains NR, 9012 and AZV, all of them from the subgenus *Leishmania* (Table), and one from the Old World, *L. (L.) major* P. The strains were serially cultured at increasing concentrations of GLIB (5 μM to 100 μM). At each concentration step, the cells remained at least three weeks. The development of resistance was monitored comparing the growth of experimental and control cells grown in parallel.

Molecular analysis - The DNA isolation from sensitive and resistant *Leishmania*, their restriction analysis, as well as the DNA labelling and hybridization conditions were carried out as described by Mendoza-León et al. (1995). The pgpA probe, a genomic fragment of 800 bp from *L. tarentolae*, present in the recombinant clone PM12, recognizes the P-glycoprotein (P-gp) family of *Leishmania* (Légaré et al. 1994). Following the digestion of the plasmid pM12 with the restriction enzyme Sac I and the fragment separation, the pgpA probe was recovered from the agarose gel and purified by the Wizard DNA purification System (Promega).

RESULTS

We have evaluated the effect of the K⁺ channel inhibitors on stationary-phase promastigote cultures of the Venezuelan strain NR. This strain was identified as a member of the *Leishmania* subgenus by the polymorphism of the ß-tubulin region (Fig. 1).

Previously, we have demonstrated that *Leishmania* strains are sensitive to K⁺ channel inhibitors (Ponte-Sucre et al. 1995). A typical dose-response curve obtained for the viability of the strain NR is illustrated in Fig. 2. Both K⁺ inhibitors, 4-AP and GLIB, affect the viability of the promastigotes along their stationary phase of growth. The LD$_{50}$ for 4-AP was 39 μM and for GLIB was even smaller, i.e., 7 μM. In addition, we have demonstrated that although the infection of the macrophage cell line J774 and peritoneal-exu-

TABLE

Designation of *Leishmania* species

Subgenus	Species[a]	Strain designation[b]	Abbreviation	Origin
Leishmania (L.)				
	mexicana	MNYC/BZ/62/M379	M379	Belize
	amazonensis	IFLA/BR/67/PH8	PH8C5 [c]	Brazil
	major	MRHO/SU/59/P-strain	P	USSR
	unknown[d]			
		MHOM/VE/72/AZV	AZV	Venezuela
		MHOM/VE/80/NR	NR	Venezuela
		MHOM/VE/90/LCP9012	9012	Venezuela
Viannia (V.)				
	braziliensis	MHOM/BR/75/M2903	M2903	Brazil
	guyanensis	MHOM/BR/75/M4147	M4147	Brazil

a: the nomenclature of *Leishmania* proposed by Lainson and Shaw (1987); *b*: the majority of these strains have been designated as reference strains by WHO; *c*: this is a cloned derivative of *L. (L.) amazonensis* stock PH8; *d*: these Venezuelan strains have been identified as members of *Leishmania* subgenus.
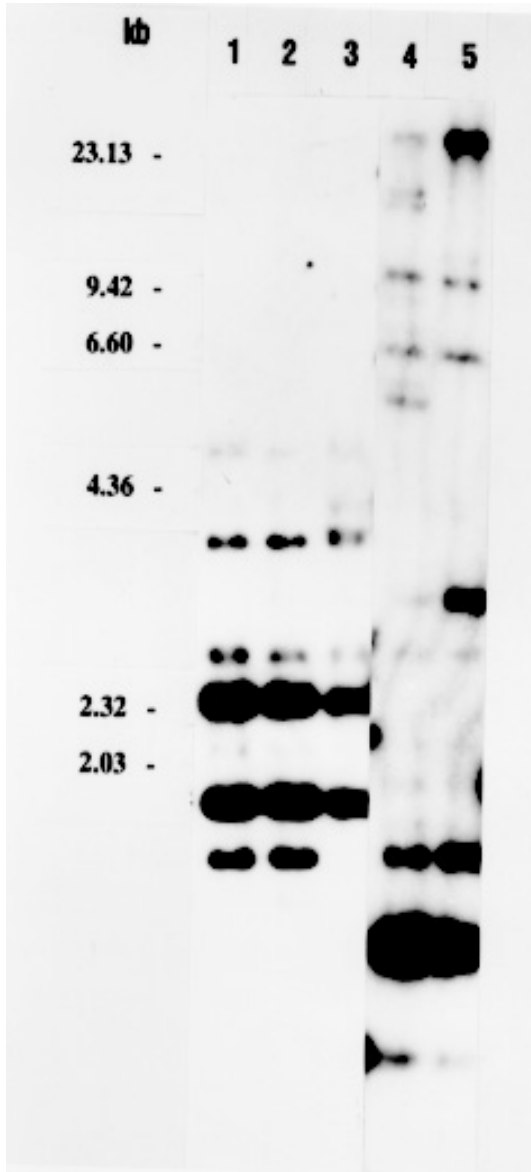
date macrophages by *L. (L.) major* is not significantly decreased by concentrations of the drugs 3-4 times smaller than the promastigote LD_{50}, 464 µM 4-AP and 10 µM GLIB for this strain, the survival of intracellular parasites decreased significantly in the presence of these drugs without affecting the viability of the macrophages (data not shown). For instance, a concentration of 3 µM GLIB, affects the viability of the macrophages less than 5%.

In view of these results and trying to understand the response of the parasite against these drugs, we further attempted to select and molecularly characterize a *Leishmania* strain resistant to GLIB (Gr). Of the three isolates of New World *Leishmania*, NR, 9012 and AZV, and the one from the Old World *L. (L.) major* P, used for the drug resistance selection, only the strain NR survived at concentrations higher than 11 µM (Gr); the rest of the isolates remained sensitive to the drug at concentrations higher than 8 µM (Gs). The maximum concentration of GLIB used to select the isolate NR(Gr) was 16 µM. Fig. 3a illustrates the behavior of the strain NR cultured at GLIB 3 µM for three consecutive weeks, and the succesful selection of the resistant strain. It can be observed that by the third week both control (Gs) and selected (Gr) cells had growing curves which were rather similar. At this stage, the parasites grown at GLIB 3 µM were serially subcultured at increasing con-
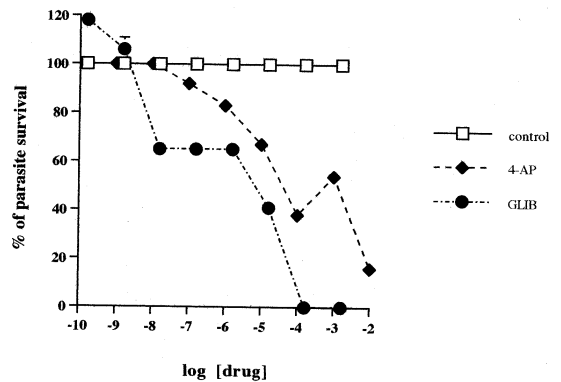

Fig. 1: *Leishmania* identification. Genomic DNA from New World *Leishmania* was digested with the endonuclease Pst I, fractionated on 1% agarose gel, and transferred to a nylon membrane. The blot was hybridized at medium stringency conditions (2x SSC, 0.1% SDS 2X Denhardt solution, 100 µg/ml calf thymus DNA, 67°C) to the *Leishmania* ß-tubulin clone pLgß4. *L. (L.) mexicana* M379 (lane 1), *L. (L.) amazonensis* PH8C5 (lane 2) *L. (V.) braziliensis* M2903 (lane 4) and *L. (V.) guyanensis* M4147 (lane 5), were used to identify the Venezuelan *L. (L.)* NR strain (Lane 3). Molecular weight markers are from the Hind III digestions of λ-DNA.

Fig. 2: effect of the ion channel inhibitors 4-aminopyridine ( ) and glibenclamide ( ) on the viability of stationary phase of the *Leishmania (L.)* NR strain. Data are presented as the mean ± standard error of the mean of 6 replicates. To estimate the fraction of free drug concentration of GLIB in the medium the following equation was used: $\alpha_{pt} = 1/[[alb_{pt}/alb_{nl}]][(1-\alpha_{nl})/(\alpha_{nl}) +1]$, where alb_{nl} and α_{nl} refer to values of concentration of albumin and the fraction of free drug in an standard condition and alb_{pt} and α_{pt} those present in the experimental medium respectively. Use of this equation assumes a molar concentration of drug far less than that of albumin, only one type of drug binding site on albumin and no cooperative binding interactions.

centrations of the drug, up to 16 μM (Fig. 3b). Fig. 3a also demonstrates the susceptibility of the sensitive parasites to GLIB 3 μM and 16 μM. The effect of GLIB 16 μM on the NR(Gr) strain, compared to the susceptibility of the NR(Gs) strain is illustrated in Fig. 3b. These results demonstrate that

the viability of the resistant strain selected in the laboratory is not impaired at this drug concentration.

An analysis of the genomic DNA of cells both of NR(Gs) and NR(Gr) was then done. DNA from both strains was digested with different restriction
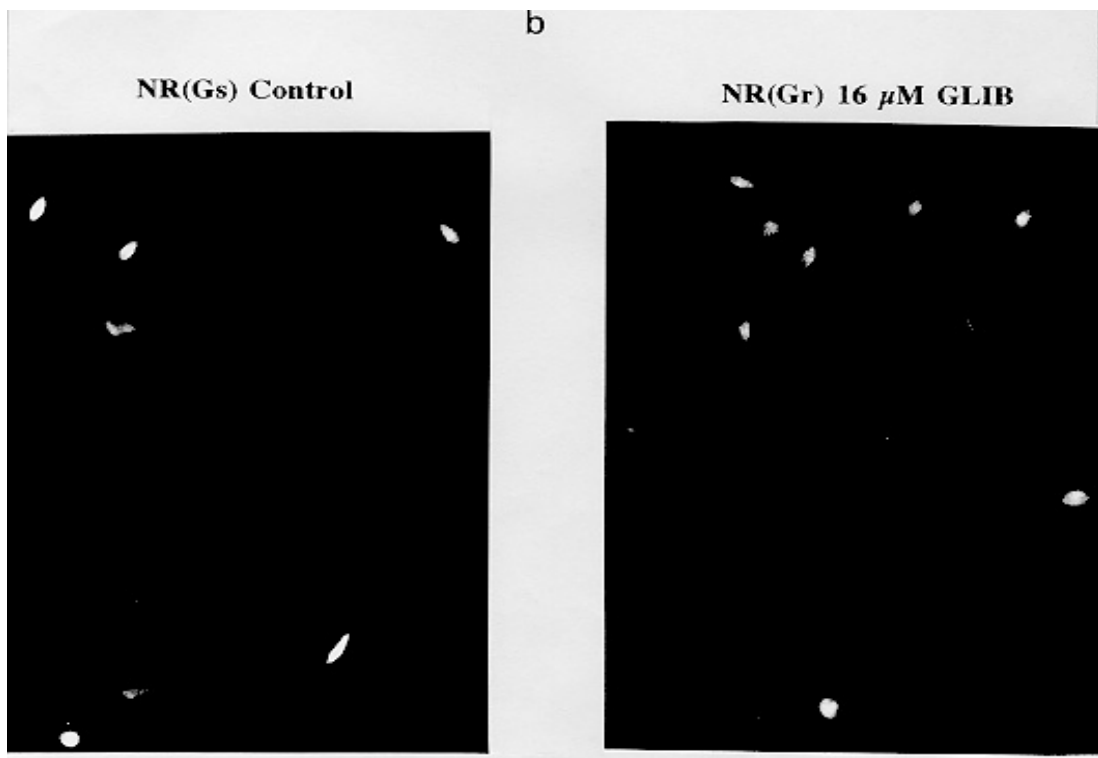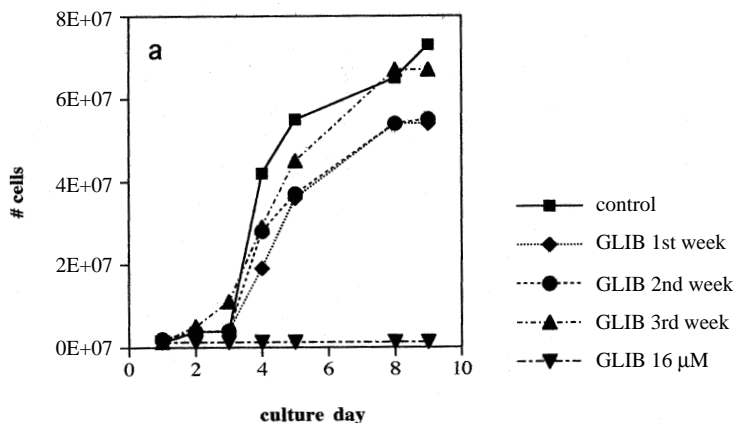

Fig. 3: sensitivity of the Venezuelan strain *Leishmania (L.)* NR strain (sensitive to glibenclamide) to glibenclamide 3 μM (   ) and 16 μM (t) and development of resistance by NR to GLIB 3 μM at week 2 (   ) and 3 (s) of subculture (a) and number of alive *Leishmania (L.)* NR cells per field in a sensitive strain NR(Gs) under control conditions and number of alive NR cells in a resistant strain NR(Gr) exposed to GLIB 16 μM (b). Magnification 400 x.

enzymes such as Bam HI, Pst I and Hind III, and hibridized at low Cot conditions to a pgpA probe from the *Leishmania* P-gp genes (related to the resistance to arsenite and antimonials). As illustrated in Fig. 4, when Pst I is used as the restriction enzyme, there is a polymorfism in the size of the fragment recognized in NR(Gs) (6.2 Kb) and NR(Gr) (4.4 Kb) by the probe. Since the same amount of genomic DNA was used, these results suggest that this fragment seems to be amplified in the strain NR(Gr).

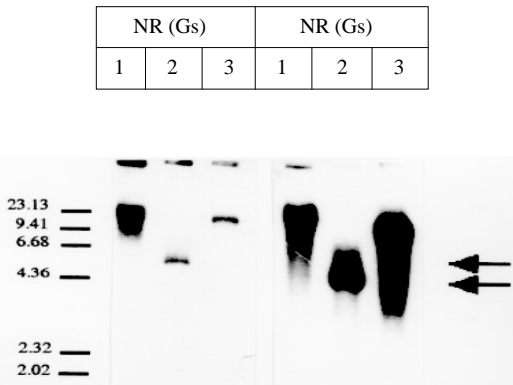| NR (Gs)			NR (Gs)		
1	2	3	1	2	3

Fig. 4: southern blot analysis of pgpA sequences in *Leishmania*. Genomic DNA from the Venezuelan *Leishmania* (*L.*) NR strain (Gs) and (Gr) was digested independently with the enzymes Bam HI (1), Pst I (2) and Hind III (3), fractionated on a 1% agarose gel and transferred to a nitrocellulose blot. The blot was hybridized to the pgpA probe under low stringency conditions. The arrows show the size polymorphism of the fragment obtained by the restriction analysis with Pst I.

DISCUSSION

The study of ion channels in parasites such as *Leishmania* is mainly justified by their biomedical importance (Backer-Grunwald 1992). Their complex life cycles, their flexibility and their ability to adapt to diverse environments, where the membrane plays an important role in maintaining the cellular homeostasis, also justify the study of the membrane proteins implicated on structures such as ion channels, pores and membrane transporters (Zilberstein & Shapira 1994). The drug 4-AP has been used extensively for identifying a high variety of voltage-gated K^+ channels (Castle et al. 1989) and GLIB which is a K^+ ATP channel blocker, has a widespread use to identify K^+ channels associated with insulin secretion (Schmid-Antomarchi et al. 1987, Panten et al. 1989). More strikingly, the sulphonylurea (GLIB-sensitive) receptor is a member of the ATP-binding cassette or traffic ATP-ase superfamily (Aguilar-Bryan et al. 1995) to which the *Leishmania* P-glycoprotein

(PgpA) has been associated (Légaré et al. 1994). The fact that both 4-AP and GLIB are deleterious to cultured promastigotes, have low DL_{50} and do not seem to impair the macrophage survival at concentrations where the survival of intracellular amastigotes decreased significantly (data not shown), reflects the importance of understanding the response and mechanism of action of these transport inhibitors. In this work we have begun to study the response of the parasite against these drugs and to acknowledge the molecular mechanisms involved in the development of GLIB resistance. For this study we have used the Venezuelan *Leishmania* (*L.*) NR strain, a member of the *Leishmania* subgenus. We have selected and began the characterization of *Leishmania* (*L.*) NR resistant to GLIB [NR(Gr)]. Thus, we found that NR(Gr) survived at concentrations higher than 11 μM as the fraction of free drug present in the medium. Its viability is not impaired at a GLIB concentration of 16 μM, although the viability of the sensitive strain, NR(Gs), is dramatically affected by this drug concentration. Striking was the strong signal found, independent of the restriction enzymes tested, for the pgpA in NR(Gr) in the Southern analysis. Since equal amounts of DNA was used, these results suggest an amplification of sequences related to pgpA in the GLIB resistant strain. The pgpA probe was originally characterized as the nucleotide binding site (nbs) of the gene family of P-glycoproteins in *Leishmania* (Ouellette et al. 1990, Légaré et al. 1994). Whether the resistance found could be related to the amplification of genes associated to P-glycoproteins is an open question. Amplification of particular gene sequences in response to drug pressure has been well documented in *Leishmania*; however, many of these amplifications are unrelated to drug resistance (Beverly 1991). Therefore is worthy to continue to explore the biochemical and molecular mechanisms which determine the response of the parasite to this drug and the development of resistance by this strain. On the other hand, there is a size polymorphism for the Pst I-fragment recognized by pgpA both in NR(Gs) (6.2 Kb) and NR(Gr) (4.4 Kb). The comparison of a partial DNA sequence of the polymorphic fragment from both strains showed more than 95% of homology between them, and around 60% homology with multidrug resistance genes from *Plasmodium falciparum*'s (not shown). We do not have yet an explanation for it; nevertheless, internal gene rearrangements has been postulated as one mechanism where by it could arise; to answer this question we are currently working on the restriction map and final DNA sequence of the fragment from both strains.

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