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Changes in the infectivity, pyruvate kinase and acid phosphatase activity and p-glycoprotein expression in glibenclamide resistant *Leishmania mexicana*

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Abstract We previously demonstrated susceptibility of *Leishmania sp.* to glibenclamide, a K^+-ATP transport blocker, which interacts with members of the superfamily of adenosine 5' triphosphate binding cassette transporters. In order to characterize the molecular differences between a sensitive *Leishmania* strain, NR(Gs), and an experimentally selected glibenclamide resistant strain, NR(Gr), specific biochemical and functional parameters have been evaluated both in the wild type and in the resistant strain. Most noteworthy, NR(Gr) exhibit an increased expression of P-glycoprotein and a decreased activity of functional key enzymes such as acid phosphatase, a prominent virulent factor of the parasite, and pyruvate kinase, a key control enzyme both for the carbohydrate and protein metabolism. The specific biochemical, metabolic and functional changes observed in the resistant strain correlated with a reduced infectivity of stationary phase NR(Gr) in J774 macrophages, and suggest a mechanism to overcome the effect of glibenclamide.

Abbreviations

ABC-transporters adenosine 5' triphosphate binding cassette transporters, *CFTR* cystic fibrosis transmembrane conductance regulator, *FDP* fructose-2,6-diphosphate, *GLIB* glibenclamide, *LDH* lactate dehydrogenase, *NR(Gs)* glibenclamide sensitive *Leishmania* NR, *NR(Gr)* glibenclamide resistant *Leishmania* NR, *PEP* phosphoenolpyruvate, *Pgp* p-glycoprotein, *SEM* standard error of the mean, *SUR* sulfonylurea receptor.

Introduction

Chemotherapy is the main therapeutic approach against diseases produced by unicellular protozoan parasites including *Leishmania* (Ullman and Carter 1995). However, the efficacy of the drugs is low since these organisms easily develop resistance against them (Borst and Ouellette 1995). Thus, the development of compounds less prone to induce drug resistance and the understanding of the mechanisms involved in the development of resistance are urgently needed.

Drug resistance is usually related to the selective overexpression of a specific P-glycoprotein (Pgp) in the cell plasma membrane, also reported in drug resistant *Leishmania* (Callahan and Beverley 1991;

Ouellette et al. 1991). Pgp could play a fundamental role in the expression of drug resistance since it extrude drugs against concentration gradients and decrease their intracellular concentration (Borst and Ouellette 1995). However, it has recently been argued that overexpression of Pgp is insufficient to explain all the related phenomena and that the evolution of drug resistance might involve additional functional and biochemical changes including ion exchange dysregulation (Roepe and Martiney 1999).

We previously demonstrated (Ponte-Sucre et al. 1998) susceptibility of various species of *Leishmania*, either cultured as promastigotes or while infecting macrophages, to glibenclamide (GLIB), a K^+-ATP transport blocker. This drug interacts with members of the superfamily of adenosine 5' triphosphate binding cassette transporters (ABC-transporters), such as the sulfonylurea receptor (SUR) (Inagaki et al. 1995), the cystic fibrosis trans-membrane conductance regulator (CFTR) (Schultz et al. 1996), and Pgp from different drug resistant cell lines (Golstein et al. 1999). In an effort to understand if the action of GLIB on *Leishmania* is targeted to Pgp, we have experimentally selected a GLIB resistant Venezuelan *Leishmania* strain NR [NR(Gr)], and initiated the molecular characterization of the mechanisms involved in the expression of this phenotype. The development of resistance to GLIB was found to be associated with the amplification of DNA sequences probably related to *ltpgpA* (Ponte-Sucre et al. 1997) from *Leishmania tarentolae* (Légaré et al. 1994) but might also involve other mechanisms. Therefore, the aim of this study was to evaluate the correlation between the expression of GLIB resistance and functional parameters, such as the infectivity of the parasite, the expression of Pgp and the activities of key enzymes such as acid phosphatase and pyruvate kinase. The results indicate that resistance to GLIB correlates with multiple biochemical and functional changes which might help to overcome the effect of GLIB.

Materials and Methods

Drugs, strains and culture conditions

The drugs were obtained from Research Biochemicals International (USA) and prepared as previously described (Ponte-Sucre et al. 1998). *Leishmania* NR(Gs), was kindly provided by Dr. Angel Hernández. It

was originally isolated in 1980 from a Venezuelan patient with tegumentary diffuse leishmaniasis and identified as a member of the *Leishmania* subgenus (Luis et al. 1998). Promastigotes of *Leishmania* NR were grown as described (Eresh et al. 1993; Mendoza-León et al. 1995). The resistant strain NR(Gr), was selected *in vitro* by successive passages of the sensitive strain grown in the presence of 16 μM GLIB as previously described (Ponte-Sucre et al. 1997) and maintained under the pressure of the drug.

Drug effect on stationary phase NR(Gr)

Aliquots of stationary phase *Leishmania* NR(Gr) (2 x 10^6 cells ml^{-1}) were exposed to 40 μM GLIB at room temperature for 4 h, in a final volume of 0.1 ml of fresh RPMI medium. At the end of the incubation time, the number of live cells was quantified by fluorescence microscopy at 495 nm after staining with fluorescein diacetate (Ponte-Sucre et al. 1997) and compared to the number of live cells in control vials of NR(Gs) incubated without GLIB.

Infectivity of NR(Gr)

The cell line J774 was grown as previously described (Ponte-Sucre et al. 1997). Cultures of 2 x 10^5 J774 ml^{-1} were infected at a parasite:macrophage ratio of 10:1 with stationary phase promastigotes from NR(Gs) or NR(Gr), in a final volume of 0.5 ml of RPMI culture medium for 4 h. Intracellular parasites were then quantified by fluorescence microscopy at 495 nm after staining with acridine orange and ethidium bromide (Ponte-Sucre et al. 1998).

Electrophoresis and Western blotting

Crude cell homogenates were separated by PAGE and transferred into nitrocellulose membranes by standard procedures. The membranes were assayed with polyclonal antibodies prepared against (1) the *Leishmania* pyruvate kinase (Anti-pyk) (1:2000) (Ponte-Sucre et al. 1993) and (2) a conserved serine-phosphorylable sequence from the N-terminal of some mammalian pyruvate kinases, (Anti-4342) (1:1000) (Hermoso, T., personal communication), as well as with monoclonal antibodies prepared against

(1) serine-phosphorylated sequences (clone PSR-45) (Anti-phosphoser) (1:1000) (Sigma), (2) *Leishmania mexicana* flagellar proteins (Anti-B7) (1:10000) (Ismach et al. 1989) and (3) P-glycoprotein, namely, (a) (C-219) (1:20) (Dako), (b) (Anti-MRP) (1:250) (Chan et al. 1997) and (c) (Anti-F4) (1:250) (Sigma). Diluted peroxidase coupled goat anti-rabbit (1:5000) or anti-mouse (1:2500) (Gibco) were used as secondary antibodies.

Acid phosphatase and pyruvate kinase activity

Acid phosphatase (EC.3.1.3.2.) activity was measured, in a volume of 0.4 ml containing 50 mM sodium acetate buffer at various pH values, 0.005 mM p-nitrophenylphosphate as the substrate and the appropriate volume of the samples. The reaction mixtures were incubated in a water bath at 37 ºC for 30 min. The reaction was stopped by the addition of 1.6 ml of 1.0 M NaOH and the reaction product was measured at 410 nm. 1 U of activity was defined as the amount of enzyme hydrolyzing 1 nmol of substrate per minute (Gottlieb and Dwyer 1981).

Pyruvate kinase (EC.2.7.1.40) activity was measured as the change in absorption of NADH at 339 nm (25 °C) due to the coupled conversion of pyruvate to lactate catalyzed by lactate dehydrogenase (EC.1.1.1.27) (LDH). The reaction solution contained 50 mM triethanolamine pH 7.2, 6 mM $MgCl_2$, 50 mM KCl, 0.42 mM NADH, 9 U LDH, 0.4 mM ADP and different concentrations of phosphoenolpyruvate (PEP), in a volume of 1 ml. 1 U of the enzyme was defined as the amount of enzyme hydrolyzing 1 µmol of substrate per minute (Ponte-Sucre et al. 1993).

Protein assay and statistical analysis

Protein concentrations were estimated by the dye-binding method (Bradford 1976) using serum albumin as standard. The data of enzyme activity are expressed as mean $\pm$ SEM of three replicates done in duplicate. Differences between the activity of the enzymes were tested for statistical significance by unpaired Student *t* test (Schefler, 1981).

Results

Effect of GLIB resistance development on Leishmania phenotype

Morphological differences between NR(Gr) and NR(Gs) were not significant and the duplication time of exponentially growing parasites remained similar, 34 and 37 h respectively. NR(Gr) was selected at 16 µM GLIB; nevertheless, the EC_{50} could not be reached even at concentrations of GLIB as high as 40 µM (Fig. 1). Finally, although the resistant parasites were still able to infect J774 macrophages, the percentage of infected macrophages was 30 % lower (18.00 ± 0.04 *vs*. 12.00 ± 0.004, $p<0.001$) than in cells infected with the sensitive strain. Overall, these data indicate that resistant NR(Gr) selected at low concentrations of GLIB are capable to survive at concentrations of the drug at least 7 times higher. They also suggest that GLIB resistance results in a decreased infectivity probably due to specific changes either in the fluidity of the membrane or the activity of specific membrane proteins (see below).

Identification of Pgp, serine phosphorylated peptides and pyruvate kinase in NR(Gs) and NR(Gr)

Phosphorylated-serine residues of proteins isolated from GLIB sensitive and GLIB resistant parasites and recognized by Anti-phosphoser are shown in Fig. 2a. At first sight both immunoblots looked rather similar; neverhteless protein bands located in the range of 50-60 kDa were preferentially recognized in NR(Gs) but not in NR(Gr) (Fig. 2a).This result suggest that post-transductional modification of proteins by phosphorylation might be altered by GLIB resistance development. With the use of Anti-pyk, a protein band around 54 kDa, similar to the sub-unit previously described for pyruvate kinase from various *Leishmania*, was recognized in NR(Gs) (Fig. 2b). This band was also recognized by Anti-4342 (arrows). A similar peptide was recognized by Anti-pyk in NR(Gr) but not by Anti-4342 indicating that the expression of GLIB resistance in NR(Gr) might correlate with changes in the expression and in the phosphorylation status of the enzyme. When the monoclonal antibody Anti-B7 was used, a peptide of 71 kDa was recognized both in NR(Gs) and in NR(Gr). Finally, different antibodies were used to identify the

Pgp from *Leishmania* NR(Gr). Only Anti-F4, an antibody whcih recognizes the N-terminal end of Pgp, was able to identify in NR(Gr) a protein band around 120 kDa (Fig. 2c), thus indicating an increase in the expression of a selective Pgp in GLIB resistant parasites.

Activity of pyruvate kinase and acid phosphatase in NR(Gs) and NR(Gr)

The activity of pyruvate kinase (Table 1) was determined at 0.4 mM ADP and various PEP concentrations. Both the NR(Gs) and NR(Gr) enzymes had sigmoidal kinetics, similar $S_{0.5}$ values of 0.333 and 0.339 mM, but different V_{max} values of 2.35 and 1.19 U mg^{-1} respectively. Addition of the allosteric modulator fructose-2,6-bisphosphate (FDP) at 10 μM transformed both kinetics into a rectangular hyperbole with a decrease in the K_m to 0.015 and 0.014 mM respectively and a 24 % increase in the V_{max} of NR(Gs) from 2.33 to 2.88 U mg^{-1}, but not in that of NR(Gr). These results indicate that resistance to GLIB might be associated with an altered sensitivity of pyruvate kinase to FDP.

The activity of acid phosphatase (Fig. 3) was determined in the membrane fraction of the cells and in the supernatant of the parasite cultures. The activity of the membrane bound acid phosphatase from NR(Gr) dropped 40 %, from 600 to 350 U mg^{-1} at pH 5.0 and 70 % in the secreted acid phosphatase, from 104 to 27 U mg^{-1}. More interestingly, the enzyme from Nr(Gs) showed a pH dependence with maximum activities expressed at pH 5.0, which was not the case with NR(Gr).

Discussion

Amplification of specific genomic regions is considered as the main mechanism sub-serving drug resistance development by certain species and organisms, including *Leishmania* (Callahan and Beverley 1991; Ouellette et al. 1991). It has been recently argued that the whole phenomenon cannot be explained only by changes in the expression of Pgp (Roepe and Martiney, 1999) and that more than one mechanism should be involved (Gueiros-Filho et al. 1995; Basselin and Robert-Guero 1998; Mbongo et al. 1998). In the present study we have demonstrated that a New World *Leishmania* strain resistant to GLIB, and which

exhibits specific DNA amplification (Ponte-Sucre et al. 1997), exhibits additional biochemical, metabolic and functional changes.

The morphology of NR(Gr) did not differ significantly from the morphology of NR(Gs) and the generation time of both strains at exponential phases of growth was similar. More interestingly, GLIB EC_{50} for NR(Gs) promastigotes is 7 µM and NR(Gr) was selected at 16 µM GLIB (Ponte-Sucre et al. 1997); nevertheless, even at 40 µM GLIB, changes in the viability of NR(Gr) were hardly seen. These data indicate that, as in other *Leishmania* species (Borst and Ouellette 1995), parasites selected at low drug concentrations are capable of surviving at much higher concentrations of the drug.

Previous data suggest that drug resistance in *Leishmania* is associated with an increased expression of Pgp. For example a group of genes belonging to the Pgp family have been characterized in Pentostan$^{@}$ resistant *Leishmania tarentolae* and *Leishmania major*. Their genetic products confer low levels of resistance to arsenite and trivalent antimonials and specifically *ltpgpA* confers low levels of resistance to oxianions and pentavalent antimonials. Its sequence predicts a structural comformation similar to other Pgp with 2 hemi proteins each one with 6 membrane segments, (Papadopolou et al. 1994). In alternative studies (Haimeur et al. 1998), *Leishmania tarentolae* promastigotes selected step by step for resistance to sodium stibogluconate and mutants resistant to antimony-containing drugs and cross-resistant to arsenite exhibit amplification of one common locus; the locus amplified was novel and did not exhibit significant homology with sequences previously described. In our case, among all the antibodies used against Pgp, only Anti-F4 was able to recognize a band of 120 kDa in NR(Gr) which was not present in NR(Gs). Species differences may account for the lack of antibody reactivity to the C-terminal end of Pgp, alternatively, the Pgp expressed in NR(Gr) might be novel, it has a lower mass and is only recognized by antibodies against the N-terminal of Pgp. This would be consistent with observations in *Plasmodium falciparum* where a Pgp with a similar molecular

weight mass, and 10 instead of 12 trans-membrane regions and only one nucleotide binding site has been described (Ullman and Carter 1995). The results presented herein suggest that the Pgp found in GLIB resistant NR(Gr) might belong to this species of Pgp.

The promastigote identity of both strains was confirmed as Anti-B7 was able to recognize a band of 71 kDa in both strains (Ismach et al. 1989). Nevertheless, NR(Gr) was still able to infect J774 macrophages but to a lower extent. Reduced virulence suggests that surface membrane functions associated with the infectivity of the parasite might be hampered by the development of GLIB resistance. In fact, alterations in membrane fluidity have been described in pentamidin and amphotericin resistant *Leishmania* (Basselin and Rober-Gero, 1998; Mbongo et al. 1998). Moreover, as has been described for the secreted enzyme from *L. donovani* and *L. amazonensis* (Basselin and Robert-Gero, 1998), we have found that the membrane bound and secreted acid phosphatase activities decreased 40 and 70 %, respectively, in NR(Gr) compared to Nr(Gs). Acid phosphatase is an enzyme preferentially located in the surface membrane of many species of *Leishmania* and usually secreted into the culture medium. It has been associated with the survival of the parasite and has also been used as a taxonomical marker (Lovelace et al 1986) and as an indicator of virulence (Katakura 1988). In fact, *Leishmania mexicana amazonensis* seems to modulate its interaction with macrophages by the phosphorylation state of secreted acid phosphatase (Vannier Santos, et al. 1995). In the results presented herein the change in the pattern of phosphorylation of protein residues, the decrease in the acid phosphatase activity and its lower pH dependence might partially explain the lower infectivity of NR(Gr); consistent with results found for *Leishmania donovani* (Singla et al 1992).

In Trypanosomatidae, the existence in the glycolysis of a branch point at the level of PEP makes pyruvate kinase play a key role in the regulation of carbohydrate and aminoacid metabolism (Cannata and Cazzulo 1984; Aguilar and Urbina 1986). With the use of Anti-pyk we were able to identify a 54000 Da peptide i.e., similar in size to the pyruvate kinase subunit described for various Trypanosomatidae, namely 54378 Da in *T. brucei* (Callens et al 1991), 54337 Da in *Trypanoplasma borelli* (Ernest et al. 1994) and

55000 Da in *Leishmania mexicana amazonensis* (LTB0016) (Ponte-Sucre et al. 1993). More interestingly, GLIB resistance was not accompanied by a change in the catalytic behavior of pyruvate kinase on its substrate; that is, the $S_{0.5}$ values were similar for NR(Gs) and NR(Gr), and yet V_{max} values were significantly decreased in NR(Gr) compared to NR(Gs). Moreover, as has been demonstrated for other strains of *Leishmania* (Van Schaftingen et al. 1985; Barnard and Pedersen 1988; Ponte-Sucre et al. 1993), FDP transformed the kinetics of the enzyme in NR(Gs) into a rectangular hyperbole thus decreasing the K_m and increasing the V_{max}. The FDP effect on NR(Gr) was restricted to a decrease in the K_m to 0.014 mM. Since FDP affected $S_{0.5}$, but not V_{max}, in NR(Gr), indicating changes in the affinity but not in the capacity of the enzyme by the allosteric regulator, and since the antibody anti-4342 against pyruvate kinase phosphorylable serine residues could not recognize the enzyme in NR(Gr), one might expect the expression in this strain of a pyruvate kinase poorly regulated by intermediate metabolites or phosphorylation and by the amount of substrate.

GLIB selectively inhibits K^+-ATP channels which are fundamental for the maintenance of the membrane potential in some types of mammalian cells. The function of these channels seems to be related to specific ATP pools derived from the glycolytic pathway via pyruvate kinase (Weiss et al. 1987). A membrane potential around –100mV has been determined in various *Leishmania* species; a P-type H^+ pump, as well as K^+ transport systems have been implied to play a role in its maintenance (Glaser et al. 1992). Also, GLIB has been shown to block the transport of low molecular weight solutes, including sugars and aminoacids, in *Plasmodium falciparum* infected erythrocytes (Kirk and Horner 1993). In a similar way, in NR(Gs) GLIB might block the transport of low molecular weight solutes, including sugars, and consequently impair the activity of pyruvate kinase. Alternatively the GLIB effect in *Leishmania* could be related to the blockade of ABC-transporters such as SUR and CFTR as has been demonstrated in other cell lines (Inagaki et al. 1995; Schultz et al. 1996). This action might reduce the cell membrane potential. As the glucose transport has been postulated to be sensitive to different membrane

potential and proton gradient antagonists in *Leishmania* (Tetaud et al. 1997), this effect might consequently impair glucose transport and indirectly the activity of pyruvate kinase.

In conclusion, the Pgp expression, the infectivity and the phosphorylation pattern are significantly changed in GLIB resistant NR(Gr). The changes observed by us suggest selection of a parasite with metabolic characteristics related to a decreased activity of acid phosphatase and a pyruvate kinase poorly regulated by intermediate metabolites and the amount of substrates. They also emphasize that GLIB resistance in *Leishmania* NR(Gr) is an event which involves, in addition to the increased expression of Pgp, complementary biochemical and functional mechanisms.

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Figure Legends

Fig. 1. Viability of wild type [NR(Gs)] and of GLIB resistant [NR(Gr)] *Leishmania* strains. Resistant *Leishmania* were experimentally selected with 16 µM GLIB and exposed to 40 µM GLIB.

Fig. 2. Western blot analysis of wild type NR(Gs) and GLIB resistant NR(Gr) *Leishmania* strains. Homogenates (5-30 µg) were separated by PAGE, transferred into nitrocellulose membranes and exposed to Anti-phosphoser (2a), Anti-pyk or anti-4342 (2b) and Anti-B7 or Anti-F4 (2c). Molecular weight markers (in kilo Dalton) are indicated on the left.

Fig. 3. Acid phosphatase activity in membrane fractions and in supernatant of NR(Gs) and NR(Gr). The activity of the enzyme was measured at various pH values as indicated in Materials and Methods.

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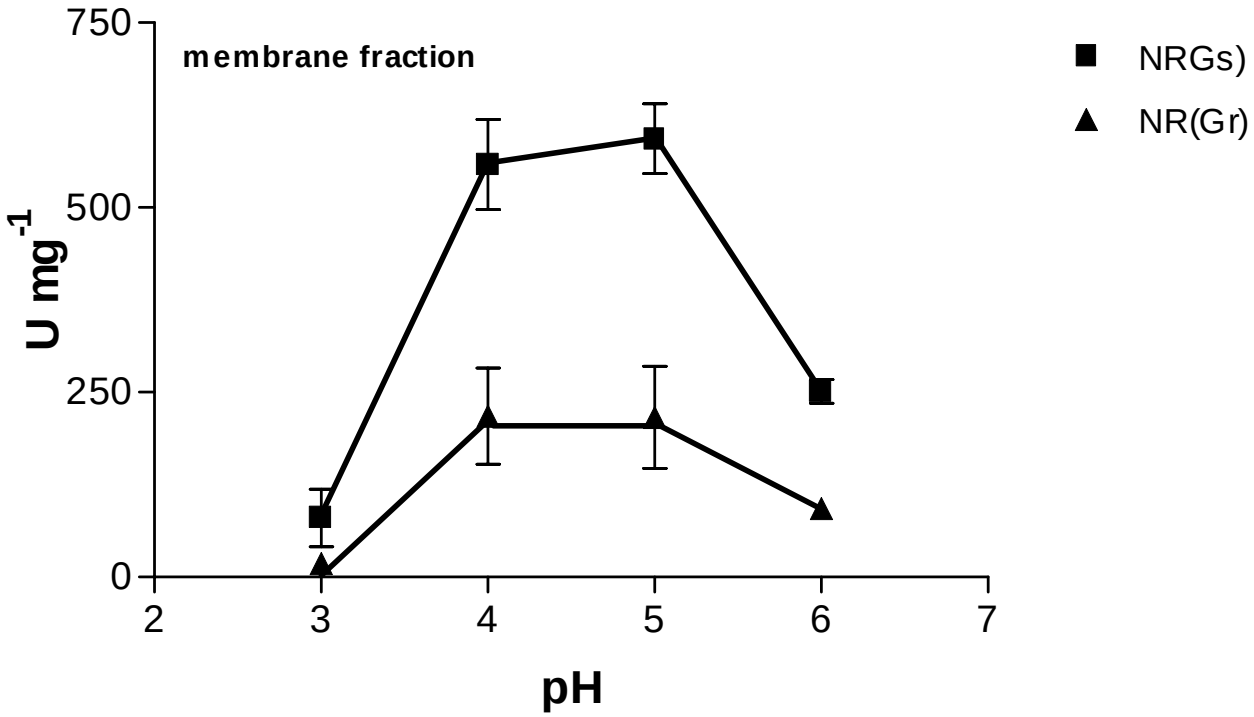
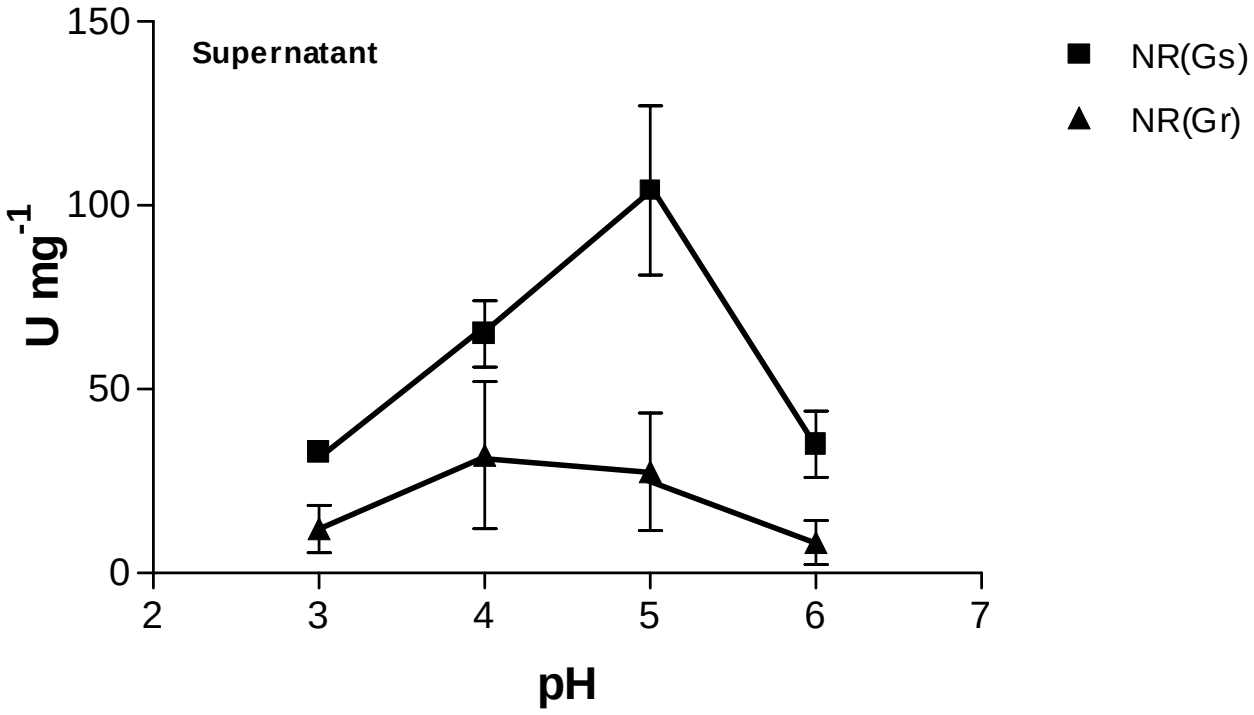
Table 1 Pyruvate kinase activity in wild type and GLIB resistant *Leishmania*

	Pyruvate kinase activity			
	-FDP		+FDP	
	K_m or $S_{0.5}$ (mM)	V_{max} (U mg^{-1})	K_m or $S_{0.5}$ (mM)	V_{max} (U mg^{-1})
NR(Gs)	0.333	2.35	0.015	2.88
NR(Gr)	0.339	1.19	0.014	1.03

Enzyme activities were determined as mentioned in Materials and Methods. The results are representative of three experiments done in duplicate. 1 U is defined as the amount of enzyme hydrolyzing 1µmol of substrate min^{-1}.


membrane fraction
750
500
250
0
U mg-1
2
3
4
5
6
7
pH
NRGs)
NR(Gr)


Supernatant
150
100
50
0
U mg-1
2
3
4
5
6
7
pH
NR(Gs)
NR(Gr)