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Short Communication

# Different Catalytic Activities of Hexokinase and Phosphofructokinase in Wild Type and Glucantime-Resistant *Leishmania* Promastigotes Appears not Causatively Related to Resistance

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## Summary

**The changes which occur in the kinetics of two enzymes, hexokinase and phosphofructokinase, involved in glycolytic metabolism have been studied here, when resistance to Glucantime is induced in vitro into *Leishmania tropica*. The results show a significant reduction (62%) in the catalytic efficiency of hexokinase in the resistant line, with the reverse occurring when this line is grown in presence of the drug (increase of 57%), whereas the catalytic efficiency in phosphofructokinase increases significantly in the resistant line (169%), especially when it is grown in presence of Glucantime (538%). Presence of the drug during growth of the resistant parasite appears to induce the synthesis of these glycolytic enzymes, whilst the alteration of its kinetic parameters suggests a modification of the glycolytic flux through the action of Glucantime. However, the determination of enzyme activity in presence of the drug does not lead to important changes in this, suggesting that Glucantime has no direct effect on hexokinase and phosphofructokinase to justify its leishmanicidal effect.**

Key words: *Leishmania tropica*, Glucantime-resistance, Wild Type, Hexokinase, Phosphofructokinase.

## Introduction

*Leishmania tropica* is a protozoan parasite causing human cutaneous leishmaniosis, which is treated with antimonial (Sb) and other drugs. More than 30 years ago, the relationship between inhibition of phosphofructokinase (PFK) and the mode of action of trivalent organic antimonials on *Schistosoma mansoni* was proved [10, 11, 16]. Later, the inhibitory effect of stibophen (trivalent organic antimonial) on PFK and aldolase of five helminth parasites was reported [22]. In all cases, glycolysis is inhibited. Gutteridge and Coombs [13] suggested a similar mechanism to explain the leishmanicidal action of antimonial drugs. Berman and co-workers [7–9] showed that Pentostam inhibited the glycolysis of *Leishmania mexicana*. In any event, the mode of action of pentavalent antimonial [Sb(V)] drugs in *Leishmania* is not fully understood. Although resistance in promastigotes might not reflect the situation found in the amastigotes, against which the therapy is directed, amastigotes derived from Sb(V)-selected promastigotes in vitro, maintained their resistance to Sb(V) [9]. The present research aimed to determine whether a difference exists in the activity and kinetic behaviour of the glycolytic enzymes hexokinase (HK) and phosphofructokinase between wild type and Glucantime-resistant *L. tropica* promastigotes. The results showed that the catalytic efficiency of these enzymes, at cellular concentrations of substrate, changes with the appearance of resistance to Glucantime in this protozoan, increasing, particularly for PFK, when the resistant parasite is grown in presence of this drug. The

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leishmanicidal action of Glucantime does not appear to be directly related to its effect on HK and PFK in *L. tropica*, as the presence of Glucantime in the reaction mixture did not modify the enzymatic velocity to any great extent.

## Materials and Methods

*L. tropica* LRC-L39 strain (LEM 2563, Montpellier, France) was used. The *L. tropica* line resistant to 10 mM Glucantime (= 1.2 mg/ml Sb(V)) (GLU-R10) has a stable resistance phenotype with a resistant index of over 1000-fold and no cross-resistance profile to structurally and functionally unrelated drugs [3]. The resistant line has a doubling time and a cell density similar to that observed for wild type cells in absence of the drug. Additionally, the pattern of proteins after SDS-PAGE analysis did not show any modification between wild type (WT) and resistant cells (results not shown). The resistant line was cultured as indicated elsewhere [3], in a medium with (RR) or without (RS) 10 mM Glucantime. For preparation of parasite extracts, promastigotes from mid-log phase of growth were used and processed to determine hexokinase (E.C. 2.7.1.1) and phosphofructokinase (E.C. 2.7.1.11) activities [1, 2]. All spectrophotometric determinations were carried out at 28°C. Enzyme activities are expressed as enzyme milliUnits (mU). One unit of HK was defined as the amount that catalysed the reduction of one micromole $NADP^+$ into NADPH per minute. One unit of PFK was defined as the amount that catalysed the oxidation of two micromoles of NADH per minute. Protein concentration was determined in the supernatant of the cell extracts [15]. Results are expressed as mean ± standard error. Statistical comparisons of data were made using the Student's *t* test. Kinetic data were fitted to the Michaelis-Menten equation using simple weighting with the non-linear regression data analysis programme ENZFITTER (Elsevier/Biosoft) written by R.J. Leatherbarrow.

## Results and Discussion

The effect of substrate concentration on HK and PFK activity and their kinetic parameters are shown in Fig. 1 and Table 1, respectively. Results show that in all cases, HK activity displays simple Michaelis-Menten kinetics. When the enzymatic activity was assayed in presence of 10 mM of Glucantime, the initial velocity was reduced by 26% for WT, 24% for RS, and 14% for RR. No significant inhibition was detected at lower drug concentrations (results not shown). Hyperbolic kinetic plots of PFK velocity against concentration of fructose 6-phosphate were obtained without evidence of sigmoidicity, as confirmed by the Hill's plots (not shown), probably due to the use of alkaline pH in the enzymatic assay [23]. No significant inhibition was detected when the PFK activity was assayed in presence of Glucantime concentrations as high as 10 mM (results not shown).

The kinetic parameters determined for the two enzymes studied in this research in *L. tropica* come within the range of values reported previously for other species of *Leishmania* [4, 5, 14]. From the results obtained, it can be seen that the resistant line presents a kinetic behaviour of HK and PFK different to the WT line.

The resistant line (RS) significantly reduces the catalytic efficiency of HK, by diminishing in particular its initial velocity, possibly by reducing the number of enzyme molecules in the protozoan. A reversion of this process occurs when the resistant line is cultured in presence of Glucantime. Although the affinity for the substrate diminishes (see Michaelis constant (Km) in Table 1), the increase (3.3-fold) in the maximal velocity (Vmax) leads to a significant increase in the catalytic ef-

**Table 1.** Changes in kinetic parameters of the glycosomal hexokinase (HK) and phosphofructokinase (PFK) in *Leishmania tropica* wild type line (WT) and resistant line grown in absence (RS) or presence (RR) of Glucantime.

| Enzyme | Parasite line | Km (mM) | Vmax (mU/mg) | Activity ratio | Catalytic efficiency | |
|---|---|---|---|---|---|---|
| | | | | $V_{0.05}$/Vmax | $V_{0.05}$/Km | Vmax/Km |
| HK | WT | 0.026 ± 0.003 | 62.4 ± 1.2 | 0.66 ± 0.06 | 1.57 ± 0.15 | 2.39 ± 0.22 |
| | RS | 0.037 ± 0.008$^{ns}$ | 38.0 ± 1.5$^{**}$ | 0.58 ± 0.10$^{ns}$ | 0.59 ± 0.10$^{**}$ | 1.03 ± 0.18$^{*}$ |
| | RR | 0.062 ± 0.005$^{**}$ ($^{*}$) | 128.3 ± 2.1$^{**}$ ($^{**}$) | 0.45 ± 0.03$^{*}$ (ns) | 0.93 ± 0.07$^{*}$ ($^{**}$) | 2.08 ± 0.14$^{ns}$ ($^{*}$) |
| PFK | WT | 4.75 ± 0.76 | 58.4 ± 2.6 | $10^{-3}$×(10.4 ± 1.2) | 0.13 ± 0.02 | 12.3 ± 1.4 |
| | RS | 3.71 ± 0.88$^{ns}$ | 98.4 ± 5.6$^{**}$ | $10^{-3}$×(1.3 ± 0.2)$^{**}$ | 0.35 ± 0.06$^{**}$ | 26.5 ± 4.8$^{ns}$ |
| | RR | 2.88 ± 0.34$^{ns}$ (ns) | 139.1 ± 3.8$^{**}$ ($^{*}$) | $10^{-3}$×(1.7 ± 0.2)$^{*}$ (ns) | 0.83 ± 0.08$^{**}$ ($^{**}$) | 48.3 ± 4.4$^{*}$ ($^{*}$) |

The kinetic parameters (Vmax and Km) were fitted to the Michaelis-Menten equation using simple weighting with the non-linear regression data analysis programme ENZFITTER (Elsevier/Biosoft) written by R.J. Leatherbarrow. $V_{0.05}$ represents the specific activity of the enzyme at 50 µM of substrate. The units of catalytic efficiency are mU/(mg.M) for HK, and mU/(mg.mM) for PFK. Data are the means ± S.E.M. of 2 to 4 experiments run in triplicate. P values refer to significance of difference between WT and RS or RR, and in brackets between RS and RR: (*) $P<0.05$; (**) $P<0.005$; (ns) not significant.

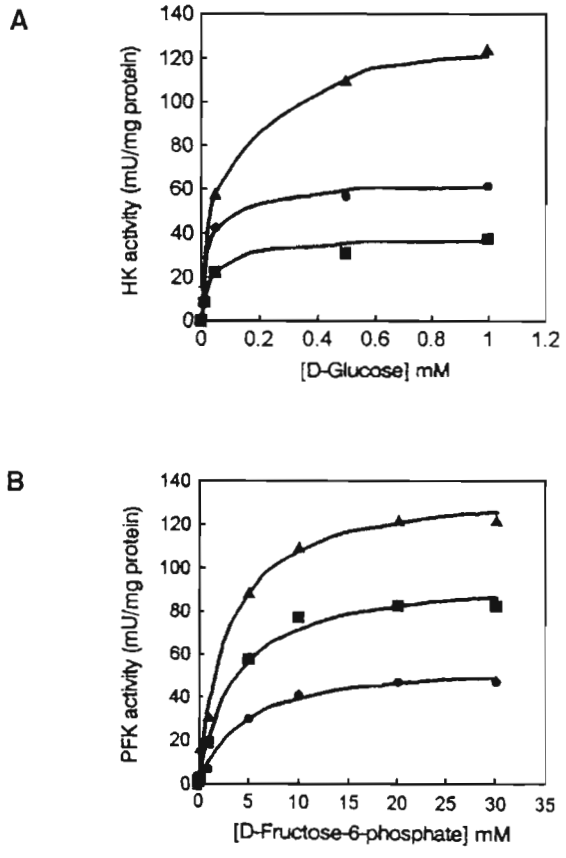


**Fig 1.** Effect of substrate concentration on hexokinase (HK) (panel **A**) and phosphofructokinase (PFK) (panel **B**) activities in *Leishmania tropica* wild type line (WT, ●) and resistant line grown in absence (RS, ■) or presence (RR, ▲) of Glucantime.

ficiency with respect to RS, particularly at saturating concentrations of substrate (Table 1). In spite of the induction of these changes in kinetic parameters of the HK by the drug, its direct effect on the enzymatic activity in vitro is not important, causing only a slight reduction in the Vmax at concentrations as 10 mM Glucantime. The action of the drug in modifying the kinetic characteristics of the enzyme may not be direct, i.e. on the molecule of the enzyme itself, but could well affect its synthesis. In this regard, Berman *et al.* [7] report a reduction in the synthesis of DNA, RNA and proteins by action of Pentostam. Furthermore, considering that the molecule of Glucantime contains a glucosyl group in its structure, it could be suggested that this slight inhibition might be due to competition with the substrate (glucose) of HK, rather than because of a direct effect of the Sb(V) on the enzyme.

With regard to the enzyme PFK, the resistant line (RS) increases its catalytic efficiency with respect to WT, at least at subsaturating concentrations of substrate – which are those which most probably occur inside the flagellate – possibly with an increase in the number of PFK molecules. It may be that this increase is related to the development of resistance by the parasite to Glucantime. The significant increase in the catalytic efficiency of PFK when the resistant line is grown in presence of Glucantime, as a response of the protozoan to the presence of the drug, could support this suggestion, although this also occurs in HK. However, the absence of any effect by this drug on the Vmax of PFK, when this is assayed in the presence of 10 mM of Glucantime, indicates that, as in the case of HK, the drug does not act directly on the enzyme. In this regard, although various compounds of Sb(III) have demonstrated their inhibitory action on PFK of helminth parasites [10, 11, 16, 22], neither Sb(V) nor Sb(III) drugs significantly inhibited HK, PFK or pyruvate kinase of *L. mexicana* [18]. But it must be borne in mind that *L. mexicana* is less sensitive to compounds Sb(V), at least to Pentostam, than other species of *Leishmania* [20]. Experiments by Roberts *et al.* [21] confirm that Sb(III) agents were more potent than the Sb(V) agents as regards antileishmanial effects. Furthermore, Dey *et al.* [12] have postulated that for the activity of Pentostam, a previous reduction of Sb(V) to Sb(III) is required.

The modification of the kinetic parameters of HK and PFK in the resistant line, especially when grown in presence of Glucantime (RR), suggests that there is a modification of the glycolytic flux in the resistant line. This modification may take place by means of a modification in the amount of enzyme or its catalytic efficiency, particularly at cellular concentrations of substrates. Likewise, this could be explained by a change in the number of glycosomes, together, perhaps, with a modification in their structural characteristics. This would concur with the research of Berman and co-workers [6–9] in which they show that the antimonium inhibits glycolysis and catabolism of the fatty acids in *Leishmania*, perhaps by altering the function or the structure of the glycosome, rather than due to a direct inhibition of the glycolytical enzymes, at least of HK, PFK and pyruvate kinase [this paper, and Adroher *et al.*, 1998, First Virtual Congress about Pharmacy, Granada, Spain]. However, it should be remembered that Berman *et al.* were working with Pentostam, whilst we are working with Glucantime and that the mechanisms of resistance and also the mechanisms of action may not be identical. In fact, this same line resistant to Glucantime does not possess a cross-resistance to Pentostam [3]. In this regard, Moreira *et al.* [17] observed that strains of *Leishmania* naturally resistant to Glucantime are sensitive to Pentostam. However, Mukhopadhyay *et al.* [19] have suggested a model for drug resistance for *Leishmania* in which Sb(V)/As(V)-containing com-

pounds, including Pentostam, are reduced intracellularly to Sb(III)/As(III), conjugated to trypanothione, and extruded by As-thiol pump. The rate-limiting step in resistance is suggested as the formation of the metalloid-thiol pump substrates, in such a way that increased synthesis of trypanothione produces resistance. Increased synthesis of the substrate rather than an increase in the number of pump molecules is a novel mechanism for drug resistance. According to this theory, it seems that the changes in the kinetics of the enzymes studied here are not the cause, but rather the effect of the acquisition of resistance by the parasite.

Further studies are needed to totally clarify the effect of Glucantime on the glycolytic metabolism of *Leishmania* and, especially, on the development of resistance by the parasites.

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