



Glibenclamide modulates glucantime activity and disposition in *Leishmania major*

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ARTICLE INFO

Article history:

Received 18 September 2008
Received in revised form 1 December 2008
Accepted 15 December 2008
Available online 25 December 2008

Keywords:

Leishmania major-glucantime
Glibenclamide-ABC transporter
Drug activity-combination therapy

ABSTRACT

A source of chemotherapeutic failure in anti-infective therapies is the active movement of drugs across membranes, through ATP-binding cassette (ABC) transporters. In fact, simultaneous administration of therapeutic drugs with ABC transporter blockers has been invoked to be the way to actively prevent the emergence of drug resistance. Herein, we demonstrate that glucantime's efficacy in decreasing the infection rate of *Leishmania*-infected macrophages is strongly enhanced when used in combination with glibenclamide, a specific blocker of ABC transporters. Intracellular ABC transporters mediate glucantime sequestration in intracellular organelles. Their selective inhibition may effectively increase the cytoplasmic concentration of glucantime and its leishmanicidal activity. Our results reveal for the first time that glibenclamide targets in *Leishmania major* a compartment associated with a multivesicular system that is simultaneously labeled by the acidic marker LysoTracker-red and may represent the organelle where antimonials are sequestered. These results constitute a proof of concept that conclusively demonstrates the potential value that combination therapy with an ABC transporter blocker may have for leishmaniasis therapy.

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1. Introduction

Chemotherapy against leishmaniasis is mainly based on the pentavalent antimonial [Sb (V)] compounds sodium stibogluconate (Pentostam®) and meglumine antimoniate (Glucantime®). In order to be active against *Leishmania* infection, Sb (V) must be reduced to trivalent antimonials [Sb (III)], the active form of the drug against the parasite (Shaked-Mishan et al., 2001; Frezard et al., 2001). However, the underlying molecular mechanism of how antimonials act against *Leishmania* infections is not fully understood. Their toxicity causes serious side-effects that often result in patients terminating their treatment, thereby contributing to the worldwide escalating frequency of *Leishmania* resistance to antimonials (Davis et al., 2004; Ouellette et al., 2004; Croft et al., 2006; Loiseau and Bories, 2006). The drug amphotericin B was introduced more recently; however, its use is not widely spread due to the cost of the treatment. Finally, since 2001 the oral drug miltefosine is also in use (Escobar et al., 2002; Davis et al., 2004). Unfortunately, single point mutations in the *Leishmania* putative miltefosine transporter can lead to resistance against miltefosine (Pérez-Victoria et al., 2006) and a gene that encodes a putative polypeptide, without similarities to known proteins, has recently been isolated from *Leishmania infantum* (Choudhury et al., 2008). Upon over-expression, this protein confers resistance against miltefosine,

but also against Sb (III), the active principle of anti-leishmanial antimonials (Choudhury et al., 2008).

In leishmaniasis, ATP-binding cassette (ABC) transporters are responsible for much of the decreased sensitivity to antimonial drugs. The augmented expression of ABC transporters enhances drug extrusion and disposition (Shaked-Mishan et al., 2001) and constitutes one of the main impediments to successful chemotherapy against *Leishmania* (El Fadili et al., 2005). Hence, the specific inhibition of ABC transporter function could be a fundamental strategy in helping to maintain effective drug concentrations within the cell. Furthermore, the simultaneous administration of the therapeutic drug with an ABC transporter blocker has been invoked to be a way to actively prevent the emergence of drug resistance and stop its increasing frequency. By blocking the transporters' activity, the efficacy of a selected drug would be either maintained or enhanced. Unfortunately, this approach has the potential drawback that resistance to the inhibitor itself might emerge (Leonard et al., 2003), and hence the risk/benefit profile of the combined use of inhibitors and drugs should be evaluated on a case-by-case basis. Still, this approach is certainly a promising avenue for developing new drugs and novel strategies against the disease that may slow down drug resistance development.

Glibenclamide (GLIB) is a sulfonylurea that inhibits ABC proteins with dissimilar functions, such as the K^+ -ATP channel associated sulfonylurea receptor (Inagaki et al., 1995), the cystic fibrosis transport regulator (Schultz et al., 1996), the ABC1 transporter of immune cells (Becq et al., 1997; Hamon et al., 1997), the P-glycoprotein (P-gp) (Golstein et al., 1999), the *Arabidopsis* multidrug resistance-related

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protein AtMRP5 (Lee et al., 2004) and the multidrug resistance-associated protein (MRP) (ABCC1) of cancer cells (Conseil et al., 2005).

The administration of the ABC transporter blocker glibenclamide in experimental *in vivo* settings increases the potency of classical drugs as glucantime [Sb (V)] when administered simultaneously. This combination therapy reduces the size of lesions in BALB/c mice infected with drug-sensitive and drug-resistant *Leishmania* (Serrano-Martin et al., 2006). However, to date, the underlying molecular mechanism eliciting this positive effect remains to be elucidated. In *Leishmania tarentolae* and *Leishmania major*, an aquaglyceroporin system is responsible for cellular uptake of trivalent reduced forms of the antimony drugs such as arsenic [As (III)] and stibogluconate [Sb (III)] (Gourbal et al., 2004). A lower activity of this molecule decreases glucantime sensitivity, and down-regulation or disruption of the uptake system, leads to drug resistance (Gourbal et al., 2004). On the other hand, once Sb (V) is reduced to Sb (III) in the cell (Shaked-Mishan et al., 2001), the reduced Sb (III) accumulates in an intracellular compartment through the function of an ABC transporter (Ouellette et al., 1998). This sequestration decreases the cytosolic concentration of Sb (III) and may be responsible of the decreased parasite sensitivity to the drug eventually detected, as less Sb (III) is then available to act against its cellular target. Over-expression of the ABC transporters located in the membrane of these intracellular compartments has been then associated with the decreased sensitivity as well as with the development of drug resistance due to the increased sequestration of Sb (III) in intracellular vacuolar compartments close to the flagellar pocket (Shaked-Mishan et al., 2001; El Fadili et al., 2005).

To test whether in *Leishmania*, glibenclamide-responsive ABC transporters play a role in drug activity, herein we analyzed *in vitro* the capacity of glibenclamide to increase the efficacy of amphotericin B and glucantime to decrease the infection rate of *Leishmania*-infected macrophages. We also investigated the intracellular location of glibenclamide. Our results constitute a proof of concept that conclusively demonstrates that combination therapy with an ABC transporter blocker may be of fundamental impact for leishmaniasis therapy.

2. Materials and methods

2.1. Cell culture

The cloned virulent *L. major* isolate MHOM/IL/81/FE/BNI was maintained by passage in BALB/c mice. Promastigotes were grown in blood agar cultures at 26 °C, 5% CO₂, 95% humidity. For the experiments described here, promastigotes were washed twice with phosphate-buffered saline (PBS) and suspended at 2×10^8 cells ml⁻¹ in Click RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Invitrogen), 2 mM L-glutamine (Biochrom, Cambridge, UK), 10 mM HEPES buffer pH 7.2 (Invitrogen), 100 µg ml⁻¹ penicillin, 160 µg ml⁻¹ gentamicin, 7.5% NaHCO₃ and 5×10^{-5} M 2-mercaptoethanol (complete medium) (Sigma–Aldrich Chemical Co, St. Louis, MO, USA).

Peritoneal macrophages were obtained from naive BALB/c mice (6–8 weeks old) (Charles River Breeding Laboratories, Davis, CA, USA). Resident peritoneal exudate cells were obtained by peritoneal washing with 5 mL of ice cold complete medium. The cells were subsequently incubated for 10 min with ice cold TAC buffer (10 mM NH₄Cl in PBS, pH 7.2) to eliminate erythrocytes, centrifuged and suspended at 2×10^6 cells ml⁻¹ in complete medium.

2.2. Location of glibenclamide within *L. major*

The fluorescent markers Bodipy Glibenclamide (Bodipy-GLIB), 4',6'-diamidino-2-phenylindole (DAPI), LysoTracker-red, MitoTrac-

ker-red and N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl) hexatrienyl) pyridinium dibromide (FM464) were obtained from Molecular Probes, Inc., (Eugene, OR, USA). Live *L. major* promastigotes (4×10^8 cells ml⁻¹) were incubated 30 min at room temperature (RT), in 200 µl PBS supplemented with 5 mM glucose. The parasites were then washed with PBS and incubated with Bodipy-GLIB at a concentration of 10 µM, 30 min at RT. The parasites were simultaneously incubated with the nuclear label DAPI at a concentration of 1.5 µg ml⁻¹, 15 min at RT or the mitochondrial marker MitoTracker-red at a concentration of 40 nM, 30 min at 4 °C. In additional experiments, the parasites were stained with Bodipy-GLIB and FM464 5 µg ml⁻¹, 30 min at 4 °C to label anterior endosomes or with Bodipy-GLIB and LysoTracker-red 1 nM, 5 min at RT to label acidic compartments. After labeling, the cells were washed with PBS, mounted in frost line slides, and analyzed by fluorescent microscopy. Images were captured by the Axioscop digital camera at 100× amplification and analyzed by Adobe Photoshop-7®. In additional experiments the parasites were pre-incubated with 10 mM sodium azide 30 min at RT before labeling with the fluorophores. Experiments were repeated at least three times.

2.3. Determination of *L. major* structure by electron microscopy

Aliquots (40 µL) of cell suspension of *L. major* (2×10^8 cells ml⁻¹) were incubated in 96-well plates alone or with glibenclamide (10 µM), or amphotericin B (5 µM) used as reference compound, for 24 h. The cells were washed three times with PBS and were further fixed in 4% paraformaldehyde in PBS for 5 d. Specimens were fixed in 1% osmium tetroxide (Sigma–Aldrich) in PBS for 2 h at RT, followed by incubation in 0.5% uranyl acetate (Sigma–Aldrich) for 1 h. Cells were dehydrated in increasing concentrations of ethanol and incubated for 1 h in propylene oxide, followed by incubation for 1 h in a 1:1 mixture of propylene oxide and Epon (Electron Microscopy Sciences, Palo Alto, USA). Specimens were embedded in Epon at 60 °C for 2 days. Post-staining of sections was done with 1% uranyl acetate for 30 min. Photographs were taken with a Zeiss EM10 transmission electron microscope at 100 Kv, and scanned images were processed using Adobe Photoshop.

2.4. *In vitro* interaction of glibenclamide with amphotericin B or glucantime against intracellular parasites

Amphotericin B, glibenclamide and glucantime were obtained from Sigma–Aldrich. *In vitro* drug interactions were assessed using a modified fixed-ratio isobologram method (Seifert and Croft, 2006). Peritoneal macrophages isolated from BALB/c mice were infected and treated in the same way as in the macrophage infection rate assay (Ponte-Sucre et al., 2006). Intracellular parasites were quantified by fluorescence microscopy at 495 nm as described previously (Ponte-Sucre et al., 1998). The predetermined EC₅₀ value of each drug helped us to choose the top concentrations of the individual drugs in the interaction assay. These concentrations were selected to ensure that the EC₅₀ for each individual drug fell near the midpoint of a six-point 2-fold dilution series. Top concentrations used were 0.2 µM for amphotericin B, 30 µM for glibenclamide, and 60 µg ml⁻¹ for glucantime Sb (V) (interaction assays for amphotericin B plus glibenclamide, or glibenclamide plus glucantime). These concentrations were used to prepare fixed-ratio solutions at ratios of 5:0, 4:1, 3:2, 2:3, 1:4, and 0:5 of amphotericin B plus glibenclamide, or glucantime plus glibenclamide; each fixed-ratio solution was further diluted five times in 2-fold dilutions. From the known concentration of amphotericin B and glibenclamide or glucantime and glibenclamide in the fixed-ratio solutions, EC₅₀ values were calculated by sigmoidal analysis using the program

Graph-pad®. For each individual drug, an EC₅₀ on its own was obtained from the fixed-ratio solutions at ratios 5:0 and 0:5. Solutions at ratios 4:1, 3:2, 2:3, and 1:4 yielded the EC₅₀ of each of the drug combinations. A Fractional Inhibitory Concentration (FIC) and a sum of FICs (Σ FICs) [FIC amphotericin B or glucantime + FIC glibenclamide] could then be calculated as follows: FIC of glucantime or amphotericin B = EC₅₀ of drug in combination/EC₅₀ of drug alone. The same was applied to glibenclamide as partner drug. Parameters, FICs and Σ FICs were calculated for all the fixed-ratio solutions and the values of FICs from each pair of drugs were used to construct the corresponding isobolograms. The values of Σ FICs were used to classify the nature of the interaction (Seifert and Croft, 2006). Mean Σ FICs listed in Table 1 are the mean values of two independent experiments. Interactions were classified as synergistic when mean Σ FICs < 0.5, as antagonistic when mean Σ FICs > 4, and as indifferent when mean Σ FICs was from 0.5 to 4 (Seifert and Croft, 2006).

2.5. Data analysis

Data on the infection rate are expressed as mean values \pm standard errors of the mean of at least three experiments in which 300 macrophages were analyzed for each drug concentration. The program Graph-pad was used to fit the data to non-linear regression and to determine the concentration that decreases the infection rate to 50%.

3. Results

3.1. Sensitivity of *L. major* to glucantime and amphotericin B

Glucantime accumulates in both stages of the parasite; however, it is not active against the promastigote stage. Indeed, the *L. major* promastigotes used in this study were not sensitive to glucantime. On the contrary, a concentration of 5 μ M of amphotericin B decreased the proliferation of *L. major* parasites by 50%, similar to what has been described in assays performed previously (Pontes-Sucre et al., 2006). Additionally, at concentrations higher than 50 μ M, amphotericin B impaired the growth of macrophages whereas glucantime had no visible effect.

It is known that *Leishmania* promastigotes are highly resistant to pentavalent antimonials in solution (Callahan et al., 1997; Pujals et al., 2008). Consequently, there is a low reliability of the tests that use Sb (V) in promastigotes. For that reason, and to test if drugs efficacy is enhanced when used in combination with glibenclamide, a specific blocker of ABC transporters we used the human relevant stage of the parasite, the amastigote. We assayed the anti-leishmanial activity of amphotericin B and glucantime in freshly isolated peritoneal macrophages infected with *L. major* for

24 h and then treated for 48 h with increasing concentrations of compounds up to 0.2 M amphotericin B and 60 μ g ml⁻¹ glucantime [Sb (V)]. Treatment of infected macrophages with the compounds resulted in a dose-dependent decrease in the infection rate. A 50% decrease in the infection rate was obtained at concentrations of 0.075 μ M (confidence interval 0.0397–0.139 μ M, r^2 0.999) for amphotericin B, and 16 μ g ml⁻¹ (confidence interval 0.2–116.5 μ g ml⁻¹, r^2 0.750), for glucantime [Sb (V)]. These data suggest that intracellular *L. major* parasites are sensitive to amphotericin B and glucantime at concentrations that are within the range normally observed for sensitive parasites.

3.2. *In vitro* interaction of glibenclamide with amphotericin B or glucantime against intracellular parasites

To test whether the administration of an ABC transporter blocker could further increase the activity of glucantime against intracellular *Leishmania* parasites, we analyzed the outcome of the combination glucantime-glibenclamide on the efficacy of the later in decreasing the infection rate of *Leishmania*-infected macrophages. As a control for this experiment we used the combination amphotericin-B-glibenclamide. The mechanism of action of amphotericin B occurs at the parasite plasma membrane and not intracellularly (Ramos et al., 1996); additionally, its disposition is different and efflux mechanisms for amphotericin B have not been described in *Leishmania*. For these reasons we did not expect to obtain a potentiation of its effect with its simultaneous administration with glibenclamide. We tested our hypothesis by comparing the results obtained in infected macrophages treated simultaneously with glucantime and glibenclamide and infected macrophages treated simultaneously with amphotericin B and glibenclamide.

To assess the *in vitro* interactions we used a modified fixed-ratio isobologram method and analyzed the data at the EC₅₀ level (Seifert and Croft, 2006). Peritoneal macrophages were infected with *L. major* for 24 h and then treated for 48 h with interaction assay mixtures for further 48 h. The results are summarized in Table 1. As expected, there was not a significant interaction between amphotericin B and glibenclamide; indeed the results indicate that in general this combination lead to an indifferent interaction at the fixed-ratios evaluated. The mean Σ FICs were between 0.72 and 2.01 except for the fixed-ratio amphotericin B: glibenclamide = 4:1 where the Σ FIC was 0.100. On the contrary, the interaction of glucantime and glibenclamide was synergistic at ratios 3:2, 2:3, and 1:4 with Σ FICs < 0.5 reaching values as low as 0.0015 (fixed-ratio glucantime:glibenclamide = 1:4). These results demonstrate that the simultaneous administration of glibenclamide increases the efficacy of glucantime, but not of amphotericin B, to decrease the infection rate of infected macrophages and suggest that the glucantime:glibenclamide combination could represent a novel strategy in the fight against *Leishmania* infection.

3.3. Location of glibenclamide within *L. major*

The drug-interaction results presented herein indicate that glibenclamide targets a pathway that increases glucantime's efficacy in killing the parasites. We further identified the intracellular location of glibenclamide in *L. major* promastigotes. The results presented in Fig. 1 suggest that the fluorescent marker Bodipy-GLIB targets an intracellular system that begins at the anterior end of the cell and runs along the parasite (Fig. 1c, g, k, o). This system is simultaneously labeled by LysoTracker-red (Fig. 1i–l). Notably, Bodipy-GLIB did not mark compartments labeled with MitoTracker-red (Fig. 1m–p) or with FM464 (Fig. 1e–h), a fluorescent marker known to tag endosomal vesicles. We also found that Bodipy-GLIB did not label nuclear compartments (Fig. 1a–d).

Table 1

Mean Σ FICs^a of the interaction between glibenclamide (GLIB) and amphotericin B (Amp B) or glibenclamide and glucantime (GLUC) to decrease the infection rate of *L. major* infected macrophages.

Fixed-ratio	AmpB:GLIB	GLUC:GLIB
4:1	0.10	>4
3:2	1.42	0.014
2:3	2.01	0.370
1:4	0.72	0.0015

^a Fractional inhibitory concentrations (FIC) and sum of FICs (Σ FICs) [FIC amphotericin B + FICgGlibenclamide, or, FIC glucantime + FICgGlibenclamide] were calculated as follows: FIC of glucantime or amphotericin B = EC₅₀ of drug in combination/EC₅₀ of drug alone. The same was applied to glibenclamide as partner drug. FICs and Σ FICs were calculated for all fixed-ratio solutions and FICs were used to construct isobolograms. Mean Σ FICs were used to classify the nature of the interaction (Seifert and Croft, 2006).

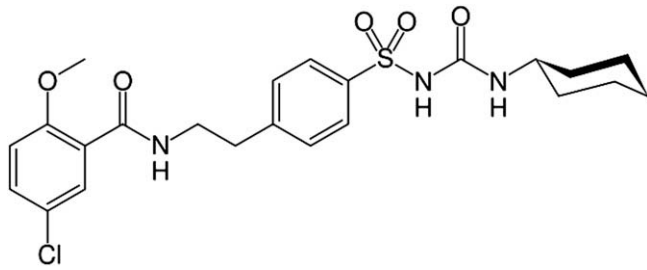


Fig. 1. Structure of glibenclamide. Systematic (International Union of Pharmacology) name, 5-chloro-*N*-[2-[4-(cyclohexylcarbamoylsulfamoyl) phenyl]ethyl]-2-methoxy-benzamide.

To evaluate the dependence of intracellular organelles labeling by GLIB on ATP/ADP levels we performed similar experiments but in *Leishmania* parasites treated for 30 min with sodium azide 10 mM. In sodium azide treated parasites the intracellular ATP/ADP ratio decreases dramatically. This decreased ATP/ADP ratio promotes the open state of ABC transporters and enhances the vesicular accumulation of substrate-inhibitors like glibenclamide by the function of specific ABC transporters. The results summarized in Fig. 2 illustrate how glibenclamide accumulation and labeling of intracellular organelles, increases significantly in sodium azide treated parasites. On the contrary, pre-incubation with sodium azide did not change the percentage of cells labeled by DAPI or by LysoTracker-red. These results thus confirm the dependence of GLIB incorporation and intracellular labeling on the cellular ATP/ADP ratio.

3.4. Determination of *L. major* structure by electron microscopy

To further analyze the effect of glibenclamide in *L. major* we studied its effect on the ultrastructure of the parasites. Untreated promastigotes (Fig. 3) displayed the typical elongated cell body, with the anterior flagellum emerging from the flagellar pocket, a single large mitochondrion containing the kinetoplast, as well as cellular structures like acidocalcisomes, endoplasmic reticulum and the Golgi complex, lipid inclusions and vacuoles. Glibenclamide (Fig. 3) did not induce dramatic morphological alterations of the parasite structure. When observed, the morphological changes were very uniform, and were mainly related to the enlargement of cellular structures like acidocalcisomes, lipid inclusions and vesicles, without disruption of the cellular structure. On the contrary, cells treated with amphotericin B showed dramatic changes including extreme vacuolization of the cytoplasm (Fig. 3).

4. Discussion

The variation in clinical response to pentostam and glucantime has been a persistent problem in *Leishmania* treatment over the past 50 years; the lack of responsiveness of the parasites to antimonials related to intrinsic differences (Croft et al., 2002; Laurent et al., 2007) but also to acquired resistance (Ponte-Sucre, 2003; Ouellette et al., 2004). This variable drug sensitivity has been explored in laboratory studies; unfortunately, it is difficult to compare results from different laboratories, as variations in assay conditions can lead to several-fold differences in activity values (Croft et al., 2002).

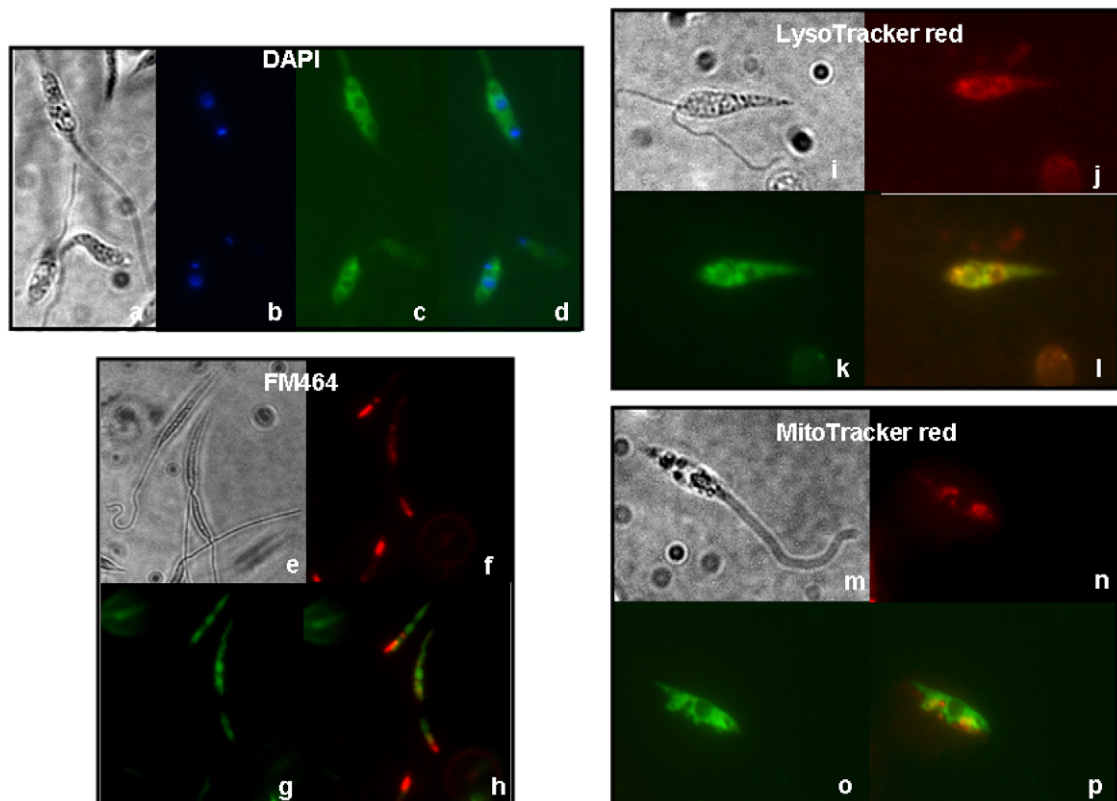


Fig. 2. Location of the fluorescent marker Bodipy-GLIB in *Leishmania major* promastigotes. (a,e,i,m) Phase contrast pictures of parasites; (c,g,k,o) parasites stained with Bodipy-GLIB, (b) DAPI, (f) FM464, (j) LysoTracker-red, and (n) MitoTracker-red. Images were captured by the Axioscop digital camera at 100 \times magnification and analyzed by Adobe Photoshop-7. Images (d,h,l,p) correspond to the overlay of images that illustrates parasites simultaneously stained with Bodipy-GLIB and the corresponding dye.

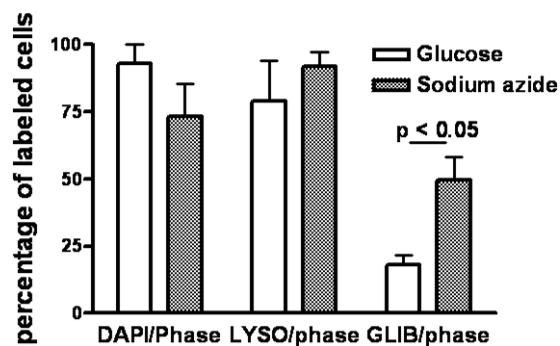


Fig. 3. Percentage of cells labeled by DAPI, LysoTracker-red, and Bodipy-GLIB in glucose and sodium azide treated parasites. Each column represents the ratio between number of cells labeled by the fluorophore to the number of cells detected in phase contrast conditions.

ABC transporters located at the plasma membrane or in the membrane of vesicular systems inside the parasite modulate the cytoplasmic levels of antimonial compounds (Ouellette et al., 1998, 2004; Shaked-Mishan et al., 2001; Singh, 2006). However, although the contribution of ABC transporters to the decreased sensitivity to drugs is not fully understood, the simultaneous administration of the therapeutic compounds with an ABC transporter blocker may be a way to actively maintain or even increase the sensitivity of the infecting *Leishmania* against the drugs and additionally to prevent the emergence of drug resistance and to stop its increasing frequency by blocking the involved ABC transporters.

In light of *in vivo* studies (Serrano-Martin et al., 2006), in which the ABC transporter blocker glibenclamide was used in combination with glucantime and found to increase its efficacy on decreasing the infection rate of *Leishmania*-infected BALB/c mice, herein we hypothesize that since the parasites decrease the cytoplasmic levels of glucantime, at least partially by sequestering the compound in intracellular organelles using an ABC transporter-mediated system (Shaked-Mishan et al., 2001; El Fadili et al., 2005), blocking these ABC transporters may help to maintain (or increase) the therapeutic cytoplasmic levels of glucantime.

Of note, glibenclamide is moderately active against *Leishmania* (Ponte-Sucre et al., 1998, 2001), and glibenclamide-resistant parasites can be selected and characterized (Ponte-Sucre et al., 1997; García et al., 2000; Silva et al., 2004; Uzcátegui et al., 2005; Machuca et al., 2006; Ponte-Sucre, 2003). Additionally, glibenclamide-resistant *Leishmania* cell lines express a temporary amplification of a fragment bearing similarity to the *ltppgA* gene (Légaré et al., 1994), which encodes the ABC transporter protein PGPA, the first MRP homolog described in *Leishmania*.

Initiatives to analyze the synergistic effect of anti-leishmanial compounds has been performed in the past using miltefosine combined with amphotericin B (Ménez et al., 2006; Seifert and Croft, 2006), as well as with other anti-leishmanial compounds like sitamaquine or sodium stibogluconate (Seifert and Croft, 2006). The unfeasibility of amphotericin B to be administered orally has hindered the success of such an approach.

Herein and for the first time we analyzed the synergistic interaction between glucantime and an ABC transporter blocker like glibenclamide. In fact, we found that the simultaneous administration of glibenclamide increases the efficacy of glucantime in decreasing the infection rate of *Leishmania*-infected macrophages; moreover, the drug combination used in our study corresponds to the 3:2 glucantime:glibenclamide ratio used in *in vivo* experiments (Serrano-Martin et al., 2006).

No synergistic effect was found when amphotericin was administered simultaneously with glibenclamide except at the combination 4:1 amphotericin B:glibenclamide. Unfortunately,

the toxicities of both drugs at this combination were greater than the additive toxicity of each of the drugs used singly. This result prevent us further discussion of these results. Of note, amphotericin B toxicity occurs through its binding to sterols in the cell membrane, formation of aqueous pores, and induction of programmed cell death (Ramos et al., 1996); intracellular trafficking has not been described for amphotericin B.

The underlying molecular mechanism of interaction leading to the enhanced efficacy of the glucantime:glibenclamide combination is not known. To further into this knowledge we began to identify the system labeled by glibenclamide. Unfortunately, colocalization experiments were not performed with antimonials since contrary to what has been developed for miltefosine (Saugar et al., 2007), fluorescent labeled glucantime is not available.

The fluorescent marker Bodipy-GLIB tagged an intracellular tubular system that begins at the anterior end of the cell, and runs along the parasite. This organelle is simultaneously labeled by LysoTracker-red. Due to the structural characteristics and cellular location, the system marked by Bodipy-GLIB and LysoTracker-red may well constitute the compartment where glucantime accumulates through ABC transporters. Since LysoTracker-red also labels acidocalcisomes, it may be possible that these are the targets of glibenclamide. However we do not think this is the case since, (1) to accumulate in acidocalcisomes drugs must be lipophilic weak bases, which is not the case for glucantime, a complex mixture of carbohydrate-antimony polymers with a dimeric structure in which two antimony atoms are linked via an oxygen, each coordinated with one molecule of gluconate, (Roberts et al., 1998) and (2) glibenclamide has little or no effect on the parasites' morphology, as examined by transmission electron microscopy (see Fig. 4), and it did not increase the appearance of acidocalcisomes like vacuoles.

Of note, the human ABC transporter MRP1 can be functionally transfected into *Arabidopsis thaliana*. The increased expression of MRP1 at the plasma membrane of the protoplast correlates with an increase in the resistance of *Arabidopsis* to Sb (III) and a decreased metalloid content in the protoplasts due to an improvement in Sb (III) efflux. Furthermore, this Sb (III) transport was sensitive to classical inhibitors of the human MRP1, such as MK571 or glibenclamide (Gayet et al., 2006). These results are in agreement with the present demonstration that the sensitivity of *L. major* parasites to glucantime is enhanced when simultaneously administered with an ABC transporter blocker like glibenclamide.

In *L. major*, pentamidine resistance is mediated, at least partially, through the increased expression of the ABC transporter pentamidine resistance protein 1 (PRP1), whose major function is to sequester pentamidine into intracellular vesicles that are later exocytosed through the flagellar pocket (Coelho et al., 2006). A similar mechanism may explain the results presented herein. Notably, the labeling by GLIB is significantly enhanced in sodium azide treated parasites. In these treated parasites the intracellular ATP/ADP ratio decreases dramatically. This decreased ATP/ADP ratio promotes the open state of ABC transporters (Ueda et al., 1999; Sauna et al., 2001) and therefore enhances the vesicular accumulation of substrate-inhibitors like glibenclamide. Our result thus confirm that intracellular compartments present in *Leishmania* express ABC transporters sensitive to GLIB; that they respond to changes in ATP/ADP levels and remain in the open state in conditions where the ratio ATP/ADP is decreased. Due to their location and characteristics, these ABC transporters may be involved in the intravesicular accumulation of glucantime.

In view of these results, it is tempting to speculate that blocking of ABC transporters located in *Leishmania* organelles impairs the accumulation of antimonials within them as well as their extrusion from the cell, thereby increasing its cytoplasmic levels and decreasing its overall cellular disposition. Better understanding of

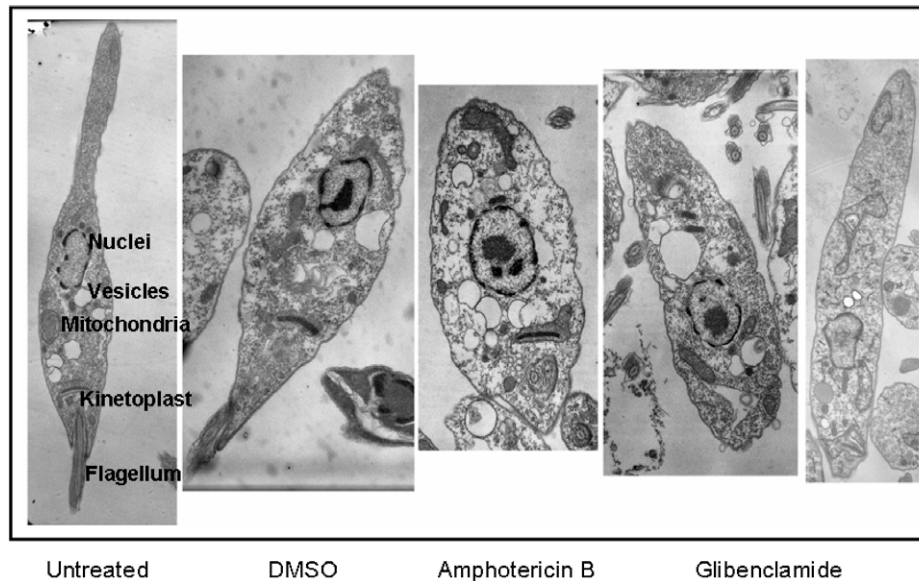


Fig. 4. Ultrastructural changes induced GLIB in leishmania promastigotes. Images were captured with a Zeiss EM10 transmission electron microscope at 100 Kv at 14,000 \times magnification and scanned images were processed using Adobe Photoshop.

the key steps involved in sequestration will be fundamental for explaining glucantime sensitivity and its experimental *in vivo* modulation by the simultaneous administration of glibenclamide in infected animals (Serrano-Martin et al., 2006).

Our results do not exclude that mammalian ABC transporters could play some role in our drug sensitivity model (Varadi et al., 2006). In fact, it has been demonstrated that drug-resistant *Leishmania donovani* parasites up-regulate the expression of ABC transporters of the host cell and this up-regulation prevents the drug from gaining access to the intracellular parasitophorous vacuole. More interestingly, inhibition of macrophage ABC transporters allows Sb (III) accumulation and parasite killing within macrophages *in vitro* and *in vivo* (Mookerjee Basu et al., 2008).

The synergistic activity of glucantime:glibenclamide herein demonstrated was done on a glucantime sensitive *L. major* strain. Additionally, the doses of glibenclamide used herein are far lower than those demonstrated to affect immunological properties of macrophages (Haskó et al., 2002). All this means that although we cannot neglect a role for macrophage ABC transporters we support the view that the ABC transporters of the parasite may have a fundamental role in the synergistic effect described herein.

In conclusion, the demonstrated synergistic interaction between glucantime and glibenclamide supports the hypothesis that combination drug therapy, by increasing the cytoplasmic drug concentrations needed and raising efficacy, may play a fundamental role in decreasing the cytotoxicity of individual drugs as well as the duration of the treatment. These results represent a proof of concept that conclusively demonstrates the potential value that combination therapy with an ABC transporter blocker may have for leishmaniasis therapy. In fact, we should point out that the importance of gene amplification or up-regulation in therapy resistant strains from the wild is still under dispute. It will be very interesting to test the results described herein on therapy resistant isolates from high endemicity regions, including areas like Bihar state or Nepal (Laurent et al., 2007). In this regard, we are presently advancing experimental settings that could allow us to test this hypothesis in isolates from latinamerican patients with a resistant phenotype.

Elucidating the molecular mechanism eliciting this positive effect would be a fundamental piece of information that unfortu-

nately is still missing. Due to the drawbacks imposed by the use of infected macrophages for furthering this goal, the use of axenic amastigotes would be desirable. However, the equivalence between axenic amastigotes and real amastigotes is far from being clearly demonstrated and waiting for that experiment could delay the possibly strong clinical implications of our work, which shows that combination drug therapy is a promising avenue to developing novel interventions that not only help to fight against infection and disease, but also actively prevent the emergence of drug resistance.

Acknowledgments

This project was supported by the Universidad Central de Venezuela Faculty of Medicine grant, FAC-MED, CI-200/2006 and Council for Research grant CDCH, PI 09-00-7084-2008, PI-09-7115-2008. We are grateful to Dr. Norys Rodriguez for supplying us with the *Leishmania major* strain.

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