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Research Article

CHARACTERIZATION OF HIGH ACTIVITY DRUG-STIMULATED *ATPase* OF MULTIDRUG RESISTANT *LEISHMANIA DONOVANI* AMASTIGOTE CELLS

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ABSTRACT

Background: The emergence of drug resistance and the limited knowledge of the mechanisms by which the parasites acquire resistance are the major obstracle for the control of leishmaniasis.
Methods: The present investigation is aimed to study multidrug resistance related ATPase activity in *in vitro* generated sodium stibogluconate (SSG) and paramomycin (PMM) resistant *Leishmania donovani* amastigote permeablized by digitonin
Results: The results of the multidrug resistance related ATPase activity in SSG and PMM resistant *L.donovani* amastigotes revealed that ATPase activities were strongly stimulated by resistance causing drugs, and these ATPase stimulation activities had broad specificities.
Conclusion: Generation of drug resistance in *L. donovani* amastigotes leads to concurrent development of drug-stimulated ATPase activity
Keywords: *Leishmania*, amastigote, ATPase, drug resistance, efflux pump.

INTRODUCTION

The trypanosomatid protozoan parasite *Leishmania* is the causative agent of kala-azar and other less severe forms of leishmaniasis [1]. Between10 to 15 million people worldwide have clinical symptom, and 400,000 new cases are diagnosed each year [2]. In the last two decades, visceral leishmaniasis especially has been widely recognized as an opportunistic infection in immunocompromised patients, particularly those who are infected with human immunodeficiency virus [3]. No effective vaccine is yet available against this parasite, and treatment relies primarily on antimony containing drugs, such as sodium stibugluconate (Pentostam) and N-methylglucamine(Glucantime) [4,5]. However, resistant parasites are now described on a frequent basis in several endemic regions [6, 7]. The second-line antileishmanials amphotericin B and miltefosine are highly effective for treatment of antimony-resistant patients but are of limited utility because of adverse reaction and high cost. A report of unresponsiveness to Ambisome in Sudanese patients of visceral leishmaniasis (VL) is alarming and indicates the emergence of AmB-resistant parasites [8].Preliminary data from a phase IV trial with miltefosine suggested a doubling of the relapse rate, indicating lower drug efficacy than in phase II and phase III trials and providing a warning about the emergence of resistance [9-11].

To serve as models for resistance, a wild strain of *Leishmania donovani* AG83 have been made *in vitro* resistant to sodium stibugluconate (SSG) and paramomycin(PMM).These mutants were independently selected in a stepwise selection *in vitro* for resistance to sodium SSG and PMM.

The ATP-binding cassette (ABC) transporters represent the biggest superfamely of proteins known, being present in all parasitic protozoa including *Leishmania* [12]. At least two

Leishmania ABC transporters are involved in drug resistance.One is PgpA(MRP1/MRPA),which is involved in resistance to arsenic and antimony containing compounds. Transfection and biochemical studies suggest that PgpA(MRP1/MRPA) recognizers metals conjugated to thiols [13,14]. The second ABC transporter is closely related to mammalian P-glycoprotein (Pgp) and confers resistance to broad spectrum of drugs by a mechanism that remains to be elucidated. The third ABCA 1 transporter, seen to be involved in the dynamic distribution of lipid species across to the membrane bilayer [14]. The forth,aquaglyceroproins (AQPs) have recently been identified in *Leishmania* as uptake systems for trivalent metalloids [15].AQPs which are channels for small neutral solutes are members of the aquaporin superfamily.All of these proteins appear to act as ATP-driven importers or exporters in the plasma membrane [16,17].

An inhibition of efflux pumps can be applied to (a) improve the transport of infflux pump antileishmanial substrate into MDR cells and (b) drug delivery. The over expression of efflux pumps such as Pgp and MRP in *Leismania* cells consequently leads to low concentrations of antileishmanial drugs inside MDR cells during leishmaniasis therapy, as a result therapeutic effects are minimized or do not occur at all.

MATERIALS AND METHODS

Materials

Standard glasswears of Borosil® were used for experimental purposes. All chemicals unless otherwise mentioned were purchased from Sigma-Aldrich (St. Louis, MO). Sodium stibogluconate (SSG) was a generous gift from Albert David Ltd. (Kolkata, India). GraphPad Prism 5.01 was used for the data analysis purposes.

Parasites and culture conditions

Promastigotes of *Leishmania donovani* clone, AG83 (MHOM/IN/83/AG83) was VL isolate obtained as a gift from Indian Institute of Chemical Biology, Council of Scientific and Industrial Research, Kolkata, India. AG83 was used to consider as reference standard strain of *L. donovani* in India. Parasites were routinely grown as promastigotes in medium 199 supplemented with 10% heat–inactivated fetal calf serum (FCS) at 24^{0}C.

Resistance selection to sodium stibogluconate and paromomycin on promastigotes and their transformation into drug resistant amastigotes

The drug sensitive AG83 promastigote cells was cultured in medium 199, in the presence of drug concentration corresponding to the 50% inhibitory concentration (IC50) of the strain. The culture was stabilized by three subcultures before increasing the drug concentration. Drug concentration was increased in such a way that the cell population was decreased approximately 20% for each batch. Finally when 90% cell population of the initial count was reduced, the phenotype so generated was plated on medium 199 agar plates in the presence of same drug concentration, and a single colony was picked for culture in medium 199 liquid media at the same drug concentration. Stability of resistance was checked at four, eight and sixteen weeks after removal from drug pressure. Evidence for the generation of drug resistant *Leishmania donovani* cells had already published [18, 19].

Generation of axenic amastigotes

Leishmania donovani amastigote forms were grown and maintained as described by Debrabant et al. [20]. Axenically grown amastigotes of *L. donovani* were maintained at 37oC in 5% CO2 /air by weekly sub-passages in MMA/20 at pH 5.5 in petri dishes [21]. Under these conditions, promastigotes differentiated to amastigotes within 168 hours. Cultures were maintained by 1:3 dilutions once in a week.

Preparation of digitonin permeabilized *Leishmania* cells

Leishmania donovani amastigote cells were collected, washed once by buffer A (140 mM NaCl, 20 mM KCl, 20 mM Tris, 1 mM EDTA, pH 7.5), and resuspended in isolation buffer (20 mM MOPS-NaOH, 0.3% BSA, 350 mM sucrose, 20 mM potassium acetate, 5 mM magnesium acetate, 1 mM EGTA, pH 7.0). Cells were permeabilized by 200 µg digitonin per mg of protein and incubated in ice for 10 minutes. After incubation, the cells were centrifuged at 6000x g for 7 minutes. Pellets were re-suspended in assay buffer.

Measurement of $Mg2^+$ -ATPase activity

The basal Mg2+-ATPase activities of Pgp MDR variants of digitonin permeabilized *L. donovani* amastigotes were measured by measuring the release of inorganic phosphate from ATP using a colorimetric method at 850 nm adapted

from Chifflet et al [22] and as described by Shapiro and Ling [23] for the measurement of ATPase activities of mammalian ABC transporters.To inhibit the contribution of other contaminating ATPase, the ATPase assay was performed in the presence of sodium azide to inhibit F0F1 –type ATPase activity, EGTA to inhibit Ca2+ -ATPase and ouabain to inhibit Na+/K+–type ATPase.These inhibitors do not interfere with ABC-dependent drug transport[24]. Digitonin permeabilized cell (about 800µg of LDC protein as determined by a modified method of Lowry [25] were incubated at 37°C in 0.1 ml of a medium containing 50mM Tris –Mes (pH 7.5),2mM EGTA ,2mM dithiothreitol ,200mM sucrose,10mM sodium azide,2mM ouabain.ATPase reaction was started by addition of 2.5mM ATPNa2 and 5mM MgSO4 for 20mins and stopped by the addition of 100µl 12% SDS.The amount of inorganic phosphate was determined immediately.ATPase activity was estimated by the difference obtained in Pi levels between 0-min(reaction stopped immediately with SDS) and 20min incubation periods.

In preliminary attempts to stimulate ATPase activity, we used antimony potassium tartarate, sodium stibugluconate, paramomycin and miltefosine. The water insoluble compounds were added in dimethylformamide. The final concentration of dimethylformamide in the assay medium was 1% (v/v). Control experiments indicated that dimethylformamide at this concentration had no appreciable effect on the ATPase activity.

Protein estimation

Total cell protein was determined by the biuret method in the presence of 0.2 % deoxycholate. One milligram of protein corresponds to 1.75×10^8 promastigote cells and 1.14×10^8 amastigote cells.

Statistical analysis

All experiments were performed in triplicate, with similar results obtained in at least three separate experiments. Statistical significance was determined by Student's t-test. Significance was considered as $P < 0.05$. GraphPad Prism 5.01 software was used for the data analysis purposes.

RESULTS

Characterization of the MDR phenotype in a *L. donovani* line

The *L. donovani* sodium SSG resistant phenotype AG83(SR) and PMM resistant phenotype AG83(PR) lines were generated *in vitro* using a stepwise selection process initiated with a concentration of 4mg Sb5+/ ml in SSG and 10µM PMM, respectively. The time required to induce resistance at a maximum concentration of 80mg Sb5+ / ml in SSG and 200 µM PMM was approximately 3 months. The resistance index which is defined as the ratio of IC_{50} of the drug in AG83 phenotype generated at maximum drug pressure and IC_{50} of drug in wild type. The resistance index (RI) of SSG and PMM in AG83 (SR) phenotype was 36 and 38, respectively. The RI of SSG and PMM in AG83 (PR) phenotype was 32 and 33 respectively. Both SSG and PMM showed cross-resistance to AG83 (SR) and AG83 (PR) phenotypes. The results of cross resistance profile of AG83 (SR) and AG83 (PR) parasites to SSG and PMM are summarized in Table I. Significant cross resistance was observed towards SSG and PMM, with a resistance index over 30. Here it is worth to note that in cellular AG83 evaluation model RI of SSG in AG83(SR) and AG83(PR) was 3 fold less than axenic AG83 evaluation model (Table I and Table II), whereas RI of PMM in AG83(SR) and AG83(PR) was 2 fold less than axenic AG83 evaluation model. Therapeutic potential of a drug is defined by its selectivity index (SI) as CC_{50}/IC_{50}. It is evident from Table II that SI of both SSG and PMM in AG83 (SR) and AG83 (PR) phenotypes were quite similar and much less than the wild-type AG83. Amphotericin B, a polyene antibiotic, appears to be highly selective in both wild and resistant AG83 strains.

Stimulation of ATPase activity by antileishmanial drug SSG and PMM

Fig 1 shows the ATPase activities, measured as inorganic phosphate (Pi) liberation from 2.5 mM mg ATP, in digitonin permeabilized AG83 (W) and AG83 (SR) and AG 83 (PR) L.donovani amastigote cells as function of ATP hydrolysis rate. ATPase activity was determined in the presence of 10mM sodium azide, an inhibitor of F0F1-ATPase, 2 mM ouabain, an inhibitor of the Na+K+ ATPase, and 2mM EGTA, an inhibitor of calcium dependent ATPase activity. These reagents have previously been shown not to interfere with P-glycoprotein-dependent drug transport [25-26]. Both SSG and PMM stimulated ATP hydrolysis rate in AG83 (W), AG83 (SR), AG83 (PR) cells. Rate of ATP hydrolysis was

Table 1: Drug sensitivity profile against *Leishmania donovani* wild-type, and drug resistant axenic amastigote cell line

	IC_{50}(mean±SD,n=4) μM[a]				
	Axenic AG83 evaluation model				
Drug	Wild-type	SSG resistant[b]	RI[c]	PMM resistant[b]	RI[c]
Amphotericin B	0.2±0.05[f]	0.21±0.05[f]	1.0	0.2360.05[f]	1.1
SSG(SbV)[c]	3.6±0.40	130±20	36	115616	32
PMM	10±2[f]	380±40[f]	38	330630[f]	33

[a]Assays are described in Materials and Methods.
[b]SSG and PMM resistant strains were generated in vitro as given in Materials and Methods.
[c]RI, Resistance Index was IC_{50} of AG83 phenotype generated at maximum drug pressure/IC_{50} of wild-type. [d]RI, Resistance Index was IC_{50} of field isolate/IC_{50} of wild-type.
[e]Values for antimonial agents are in mg Sb/ml.
[f]$p<0.001$, significant difference compared with SSG.
[g]$p<0.5$, no significant difference compared with SSG.
[h]$p<0.5$, no significant difference compared with SSG.

Table 2: Drug sensitivity profile against *Leishmania donovani* wild-type and drug resistant intracellular amastigote cell lines

	IC_{50}(mean±SD for atleast 4 replicates) μM[a]								
	Cellular AG83 evaluation model								Cytotoxicity CC_{50} (μM)(Macrophage cells)
Drug	Wild-type	SI[f]	SSG resistant[b]	RI[c]	SI[f]	PMM resistant[b]	RI[c]	SI[f]	
Amphotericin B	0.15±0.05[g]	93	0.2±0.05[f]	1.3	70	0.20±0.05[f]	1.3	68	14±2.1[h]
SSG(SbV)[c]	1.6±0.20	17	18.1±3[g]	11.2	1.5	17.3±2.60	10.8	1.6	27±3.9
PMM	8±2[g]	31	125±15[g]	15.6	2	115±13[g]	14.4	2.1	248±32[g]

[a]Assays are described in Materials and Methods.
[b]SSG and PMM resistant strains were generated in vitro as given in Materials and Methods.
[c]RI, Resistance Index was IC_{50} of AG83 phenotype generated at maximum drug pressure/IC_{50} of wild-type.
[d]RI, Resistance Index was IC_{50} of field isolate/IC_{50} of wild-type.
[e]Values for antimonial agents are in mg Sb/ml.
[f]SI, Selectivity Index was CC_{50}/IC_{50}.
[g]$p<0.001$, significant difference compared with SSG.
[h]$p<0.5$, no significant difference compared with SSG

Table 3: Effect of divalent cations on ATPase activity of the digitonin permeabilized AG83 (SR) cells

Divalent cations	% Control [a] (n=3)
None	6 ± 1
$MgCl_2$ (2.5 mM)	100± 11
$MnCl_2$ (2.5 mM)	8 ± 2
$CaCl_2$ (2.5mM)	5 ±1
$ZnCl_2$ (2.5 mM)	1 ± 1

[a]ATPase activity was assayed at 37°C colorimetrically from the release of inorganic phosphate as described in Materials and Methods. Activity is expressed as the difference between the antimonite-stimulated and the basal rates.

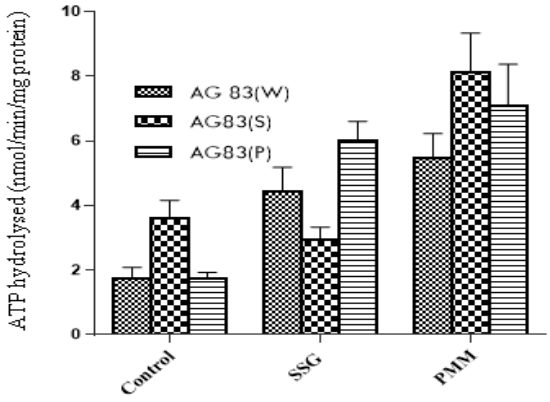

Fig 1: ATPase activity of sensitive AG83 (W) and multidrug resistant AG83 (SR) and AG83 (PR) *Leishmania donovani* amastigote digitonin permeabilized cells. The bars indicate the rate of ATP hydrolysis in absence of drug (CTRL), with 100 µM SSG, 100 µM PMM in the incubation medium. Values are means ± S.E.M. (n=3).

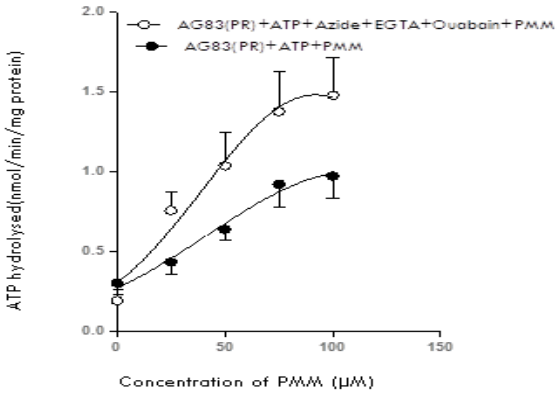

Fig.2: Effect of Azide, EGTA and ouabain on PMM stimulated ATPase activity in digitonin permeabilized L.donovani amastigote cells. Values are means ± S.E.M.(n=3).

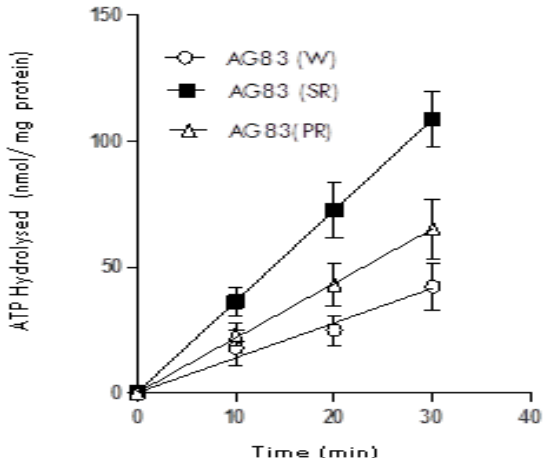

Fig 3: Time dependence of ATRase activity of digitonin permeabilized AG83 (W), AG83 (SR) and AG83(PR) *Leishmania donovani* amastigote. Aliquots containing 800 µg of protein from AG83(W), AG83(SR) and AG83 (PR) were assayed for Mg^{+2}-ATPase activity various times as described in Materials and Methods. ATPase activity is expressed as Pi released and is presented as means ± S.E.M. (n=3).

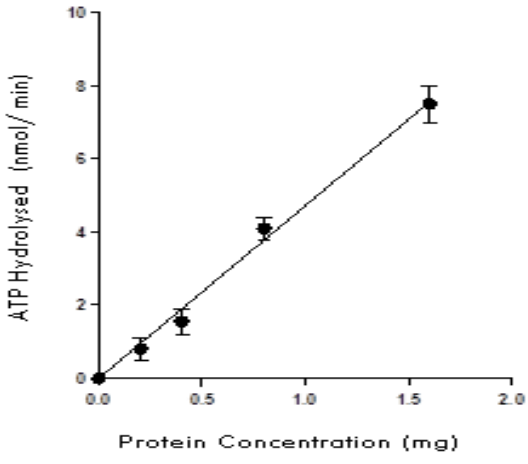

Fig.4: Protein dependence of ATPase activity from AG83 (W) digitonin permeabilized *Leishmania donovani* amastigote cells. Aliquotes containing 0.2 to 1.6 mg protein from AG83 (W) cells was assayed for Mg^{++}-ATPase activity at various protein concentrations as described materials ansa method. ATPase activity is expressed as Pi released and is presented as means ± S.E.M (n=3)

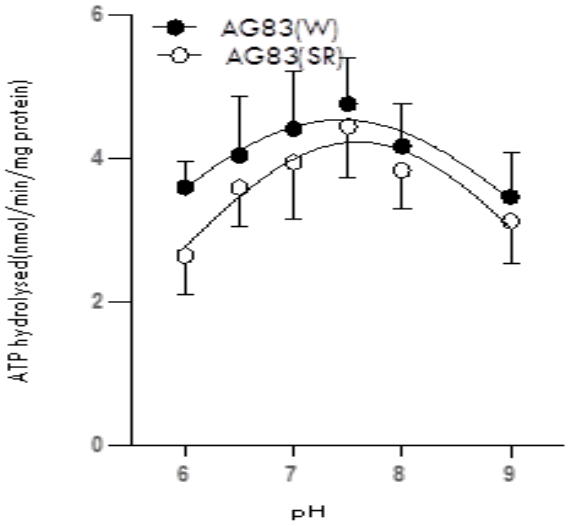

Fig. 5: Effect of pH on the Mg^{2+}-ATPase activity of digitonin permeabilized *L.donovani* amastigote cells. ATPase activity was measured in Mg^{2+}-ATPase buffer containing either 50mM Mes (6 and 6.5), 50 mM Mops (pH 7 and 7.5) or 50mM Tris (pH 8 and 9). ATP hydrolysis is presented as (nmol/min/mg protein) measured under standard conditions (i.e, 50mM Tris, pH 7.2). Data are presented as means ± S.E.M. (n=3).

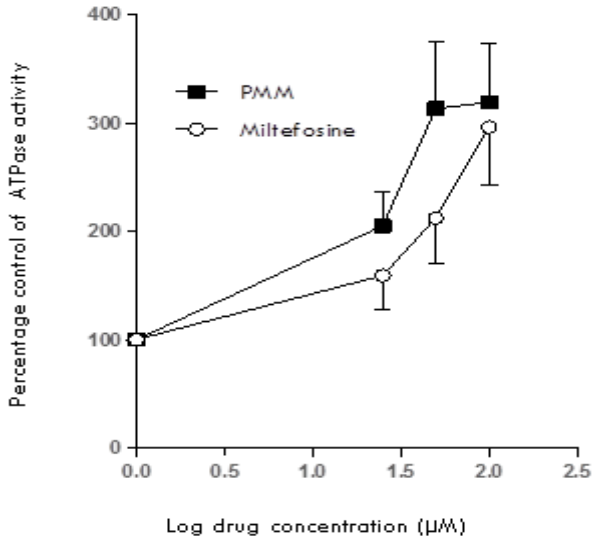

Fig. 6: Effects of drugs on MDR ATPase activity in digitonin permeabilized AG83 (PR) cell . The ATPase activity was measured in the presence of increasing concentration of paramomycin (PMM), miltefosine (MIL). Data are expressed as percent control ATPase activity (means± S. E.M, n=3) measured in the absence of drug.

found to be maximum by PMM in AG83 (W), AG83(S) and AG83 (P) cells. The appearance of the SSG and PMM-stimulated ATPase activity in the AG83 (SR) and AG83 (PR) cells correlated with MDR detected in these strains.

Summarizing this section, we found a large ATPase activity in digitonin permeabilized MDR *Leishmania* amastigote cell

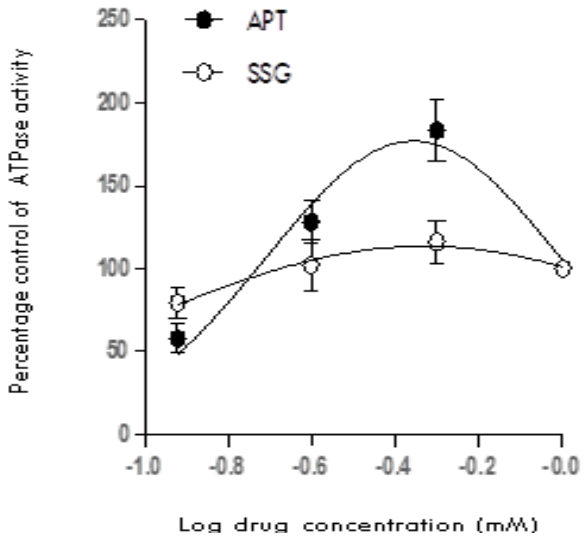

Fig 7: Effects of drug and chemosensitizer on MDR ATPase activity in digitonin permeabilized cell. The ATPase activity was measured in the presence of increasing concentration antimony potassium tartarate (APT), sodium stibugluconate (SSG,). Data are expressed as percent control ATPase activity (means± S. E.M, n=3) measured in the absence of drug or chemosensitizer.

lines that was not due to Ca++-ATPase, Na+K+-ATPase,ecto-ATPase [27], or mitochondrial F0F1-ATPase. The specific ATPase activity is similar to that reported for human P-glycoprotein expressed in Sf9 cells [28].

Effect of sodium azide, EGTA, time and pH on ATPase activity

The dependence of the rate of hydrolysis on sodium azide (10mM) and EGTA (2mM) in presence of varying PMM concentration has been shown in Fig7. MDR proteins has been reported to have a drug-stimulated ATPase activity [29], and hence drug transport and ATP hydrolysis are considered to be coupled directly. In AG83 (PR) cell, rate of ATP hydrolysis had increased in presence of increasing PMM concentration. Addition of sodium azide and EGTA in the assay medium resulted in substantial stimulation of ATP hydrolysis rate (Fig 7). Stimulation of ATP hydrolysis rate in presence of sodium azide and EGTA appears to be due to specific MRP protein related drug-stimulated ATPase activity, and it is also evident that F0F1-ATPase and Ca++ -ATPase were not stimulated by drug. ATP hydrolysis by MDR protein in AG83 (W), AG83 (SR) and AG83 (PR) cells increased linearly with time (Fig 3). The ATP hydrolysis was also propotional to the amount of digitonin permeabilized cell added (Fig 4). No significant loss of activity was observed

when the digitonin permeablized cell was incubated at 4°C for 4 h prior to assay. pH optimum for ATPase activity in AG83(W) or AG83(SR) appears to be in between pH 7 and 8 (Fig 5). At pH higher than 8 and lower than 7, the hydrolytic activity was reduced. Based on these observations, the ATPase assay was routinely performed at pH 7.5 at 37°C.

Effect of paramomycin and miltefosine on ATPase activity of digitonin permeablized AG83 (PR) cells appears to be strongly stimulated by PMM and miltefosine(MIL) (Fig. 6). Thus, ATPase activity of AG83 (PR) has specificity to another structurally unrelated MIL, an antileishmanial drug.

Effect of oxyanions on ATPase activity of digitonin permeabilized AG83 (SR) cells

The ATPase activity of ArsA protein has been shown to be stimulated by arsenite and antimonite [30]. In our observation, similar oxianion stimulation has been observed in digitonin permeablized AG83 (SR) cells (Fig. 7). SSG had less ATPase stimulating effect compared to antimony potassium tartarate (APA). Stimulation by antimonite was 4 fold higher than arsenite (data not presented). SSG and APT stimulated ATPase activity exhibited an absolute requirement for Mg2+ (Table III). Neither Ca2+ nor Mn2+ could substitute for Mg2+, Zn2+ was inhibitory.

DISCUSSION

Resistance of parasitic protozoa such as *Leishmania* to antileishmanial drugs continues to escalate in developing countries. Treatment programs for human leishmaniasis are still based on pentavalent antimonials, paramomycin, miltefosine and amphotericin B, but resistance to these compounds has been a persistant problem. In many instances, resistance of the parasite is due to over-expressed ABC efflux pumps [31], and the ABC transporter MRPA (PgpA) is implicated in the antimony resistance [32]. The second class of ABC proteins identified in *Leishmania* parasites are the transporters with a higher similarity to mammalian P-glycoproteins that confer a multidrug resistance(MDR) phenotype similar to that observed in cancer cells. *Leishmania* mdr1 (Lmdr1)-like gene have been detected in *L. donovani* [33] and other *Leishmania* spp. All of these parasite lines were stepwise selected with the antileishmanial drug and presented an MDR phenotype, with a cross-resistance profile to non-related hydrophobic drugs such as paramomycin, daunomycin,vinblastine, adriamycin and doxorubicin[33-36]. Resistance to miltefosine has been observed in cancer cells as well as in *L. tropica*, the later involving a Pgp-like transporter [37]. Paramomycin is an aminoglycoside and the mechanism of resistance in bacteria most commonly involves a mutation in the small subunit ribosomal RNA gene [38]. However, the resistance mechanism described in *L. tropica* is not due to single base-pair mutations [39]. Paramomycin resistance in *L. donovani* promastigotes has been related rather to decreased drug uptake, probably as a consequence of altered membrane composition [40].

We have obtained a highly multidrug resistant AG83(SR) and AG83(PR) *L. donovani* cell lines which showed 32 to 38-fold resistance index (RI)(Table I). These cell lines grow well in suspension culture, providing a good source of *in situ* plasma membranes enrichd in MDR P-glycoproteins for enzymatic studies. In this report we describe charactyeristics of membrane-bound MDR P-glycoproteins ATPase activity, and we had established that for preliminary characterization of plasmamembrane P-glycoproteins ATPase activity, digitonin permeabilized cell was the most facile source of plasma membrane-bound ATPase [27].

Conditions were established to measure P-glycoprotein ATPase activity in digitonin permeabilized cell preparations Ecto-ATPase was eliminated by the use of 2 mM ouabain and omission of Na+ and K+ ions, and Ca++ATPase was eliminated by use of EGTA. PMM stimulations of ATPase activity in AG83 (PR) cell in presence of sodium azide, EGTA and ouabain, suggested the specificity of drug stimulated P-glycoprotein in the assay system. ATPase activity showed a good correlation with RI of AG83 (SR) and AG83 (PR) cell lines (Fig. 1). AG83 (W) cells, from which AG83 (SR) and AG83 (PR) was derived, stimulated by SSG and PMM. PMM showed maximum stimulation followed by SSG. Stimulation of ATPase activity by SSG and PMM in AG83 (W) cell suggest the presence of constituteive P-glycoproteins. To be an enzyme, plasmamembrane-bound ATPase activity showed linearity with time and protein content (Fig. 3, Fig. 4) and pH maxima in the range of 7 to 8 (Fig. 5).

The current model for MDR, which envisions P-glycoproteins as an ATP-dependent drug efflux pump, predicts that ATP hydrolysis is directly coupled to drug transport. The ATPase activities of several other transporter ATPase are directly

regulated by the transported substrates, e.g. the Na+/K+- and Ca2+-ATPase[41], the Cl-ATPase[42] and the arsenite/ antimonite-ATPase [43] and also various organic molecule ATP-dependent transporters such as the bacterial histidine [44] and maltose[45] permeases and the erythrocyte glutathione disulfite transporter[46]. In these proteins, significant ATP hydrolysis occurs only when the transported substrate is presented to the enzyme and in the case of histidine and maltose permeases, only when the substrate is concurrently transported across the membrane. If MDR P-glycoproteins from AG83 (SR) and AG83 (PR) cells behaves similarly, drugs and other transportable molecules might be expected to have same stimulating effect on ATPase activity, as has been observed in our work.

CONCLUSION

In our study, transportable drugs PMM, MIL in AG83 (PR) cell (Fig. 6) and antimony potassium tartarate (APT), SSG in AG83 (SR) cell (Fig. 7) showed strong stimulation of ATPase activity. A major finding of this study is that *Leishmania* drug resistant cells confer cross-resistance to antileishmanial drugs, and the same phenomenon have also been observed in drug stimulated ATPase activity. Thus, drug resistant *Leishmania* phenotypes appears to have broad drug-like substrate specificity in ATP-driven efflux pumps.

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