International Journal for Parasitology 41 (2011) 1311–1321

Contents lists available at SciVerse ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Characterisation of antimony-resistant *Leishmania donovani* isolates: Biochemical and biophysical studies and interaction with host cells

Rupkatha Mukhopadhyay [a], Sandip Mukherjee [a], Budhaditya Mukherjee [a], Kshudiram Naskar [a], Dinesh Mondal [e], Saskia Decuypere [d], Bart Ostyn [d], Vijay Kumar Prajapati [b], Shyam Sundar [b], Jean Claude Dujardin [c], Syamal Roy [a,*]

[a] *Indian Institute of Chemical Biology, Council of Scientific and Industrial Research, Kolkata, India*
[b] *Institute of Medical Sciences, Banaras Hindu University, Varanasi, India*
[c] *Department of Biomedical Sciences, Antwerp University, Antwerp, Belgium*
[d] *Institute of Tropical Medicine, Antwerp, Belgium*
[e] *International Centre for Diarrhoeal Disease Research, Dacca, Bangladesh*

ARTICLE INFO

Article history:
Received 17 May 2011
Received in revised form 8 July 2011
Accepted 27 July 2011
Available online 7 September 2011

Keywords:
Leishmania donovani
Antimony resistance
Thiol metabolism
Glycoconjugates
Membrane fluidity
IL-10
ABC transporter

ABSTRACT

Recent clinical isolates of *Leishmania donovani* from the hyperendemic zone of Bihar were characterised in vitro in terms of their sensitivity towards sodium stibogluconate in a macrophage culture system. The resulting half maximal effective concentration (EC_{50}) values were compared with those of known sensitive isolates. Fifteen of the isolates showed decreased sensitivity towards SSG with an average EC_{50} of 25.7 ± 4.5 μg/ml pentavalent antimony (defined as antimony resistant), whereas nine showed considerable sensitivity with an average EC_{50} of 4.6 ± 1.7 μg/ml (defined as antimony sensitive). Out of those nine, seven were recent clinical isolates and the remaining two were known sensitive isolates. Compared with the antimony sensitive, resistant isolates showed enhanced expression of thiol metabolising enzymes in varying degrees coupled with increased intracellular non-protein thiol content, decreased fluorescence anisotropy (inversely proportional with membrane fluidity) and over-expression of the terminal glycoconjugates (*N*-acetyl-D-galactosaminyl residue). Macrophages infected with resistant but not with sensitive showed up-regulation of the ATP Binding Cassette transporter multidrug resistance protein 1 and permeability glycoprotein, while the supernatant contained abundant IL-10. The above results reinforce the notion that antimony resistant parasites have undergone a number of biochemical and biophysical changes as part of their adaptation to ensure their survival in the host.

1. Introduction

Visceral leishmaniasis (VL) or kala-azar is widening its base on most continents. An additional problem is the evolution of resistance to commonly used drugs, apart from other risk factors such as immunosuppression or human-made and environmental changes (Dujardin, 2006). For the treatment of VL or kala-azar, the pentavalent antimonial (Sb(V)) drug urea stibamine was introduced in the Indian subcontinent almost eight decades ago (Brahmachari, 1922). But the early success that the Sb(V)s had attained started to dwindle with time, even with increasing dose and duration of treatment (Croft et al., 2006). Treatment failure was associated with the emergence of drug resistance on the Indian subcontinent. As a result, it was abandoned for clinical use primarily in the epicentre of the disease, Bihar in India. However, the mechanism of resistance is still far from clear as clinical resistance to antimony is different to in vitro antimony sensitivity (Sb-S) in *Leishmania* (Ibrahim et al., 1994; Sharief et al., 2006). Decrease of drug concentration within the parasite, either by reducing drug uptake or by increasing efflux/sequestration of the drug, constitutes the primary mechanism of antimonial resistance (Sb-R); other potential resistance mechanisms include the inability to reduce Sb(V) to trivalent antimony (Sb(III)), decreased Sb(III) target in cells, or increased Sb(III) detoxification (Decuypere et al., 2005). All of these biochemical events are catalysed either by the antimony transporter genes or thiol metabolising genes. Different types of ATP Binding Cassette (ABC) transporters are known to be responsible for multi-drug resistance (MDR). MDR-related proteins (MRPA or PGPA) have also been reported to be amplified in various species of *Leishmania* in response to different drugs under laboratory conditions (Fadili et al., 2005). Thiol (namely, cysteine, glutathione (GSH), spermidine and trypanothione (TSH)) metabolism plays a central role in the maintenance of an intracellular reducing environment so that the cell can defend itself against the damage

* Corresponding author. Address: Indian Institute of Chemical Biology, 4, Raja S.C. Mullick Road, Jadavpur, Kolkata 700032, India. Tel.: +91 33 24990395.
E-mail address: sroy@iicb.res.in (S. Roy).

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doi:10.1016/j.ijpara.2011.07.013

caused by oxidative stress inside the macrophage (MΦ) brought about by oxidants, certain heavy metals and possibly xenobiotics (Wyllie et al., 2004). There is a report of variable thiol content in Indian field isolates (Mandal et al., 2007). An elegant investigation with Sb-R and Sb-S *Leishmania donovani* isolates showed that there is a great deal of difference not only in fatty acid metabolism, but also in the level of unsaturated fatty acids (t'Kindt et al., 2010). It is tempting to speculate that an increase in unsaturated fatty acids alters membrane fluidity in Sb-R isolates. A recent study has shown that Sb-S and Sb-R *L. donovani* isolates differentially regulate activation of dendritic cell (DC) maturation in response to SSG and infection with the resistant isolates induces more IL-10 generation from DCs (Haldar et al., 2010).

This study focuses on the biochemical and biophysical analyses of recent clinical isolates from a hyperendemic zone of Bihar, India and compares the results with those in known SSG-sensitive isolates. It shows that the Sb-R phenotype, compared with the Sb-S one, is associated with a wide variety of biochemical and biophysical changes and their interactions with host cells. To our knowledge this is the first report in which a number of biochemical and biophysical methods have been used to characterise a number of recent clinical isolates which were not previously reported.

2. Materials and methods

2.1. Animals

BALB/c mice (*Mus musculus*) and golden hamsters (*Mesocricetus auratus*) were maintained and bred under pathogen-free conditions. Use of both mice and hamsters was approved by the Institutional Animal Ethics Committees of Indian Institute of Chemical Biology, India. All animal experimentations were performed according to the National Regulatory Guidelines issued by CPSEA (Committee for the Purpose of Supervision of Experiments on Animals), Ministry of Environment and Forest, Government of India.

2.2. Isolation of peritoneal exudate cells (PECs)

PECs were harvested from BALB/c mice by lavage, 48 h after i.p. injection of 2% (w/v) soluble starch (SIGMA) (Basu et al., 2006). PECs were plated on 60 mm tissue culture petri dishes, 24-well and 6-well plates (Nunc) at a density of 1×10^6, 1.5×10^6 and 3×10^6 cells, respectively, as well as on sterile 22 mm square coverslips (Bluestar, India) in 35 mm disposable petriplates (Tarsons, India) at a density of 1×10^5/coverslip in RPMI 1640 medium (SIGMA) supplemented with 10% heat inactivated FBS (SIGMA)/100 IU/ml of penicillin/100 μg/ml of streptomycin (GIBCO) i.e. RPMI complete medium. The cells were left to adhere for 48 h at 37 °C under 5% CO_2 before infection. For convenience the adherent PECs were defined as MΦs.

2.3. Parasites and infection

Promastigotes of *L. donovani* isolates were harvested by transformation of amastigotes from the splenic aspirate of Kala-azar patients. The protocol was approved by the Ethical Committee of the Institute of Medical Sciences, Banaras Hindu University, India. Informed written consent was obtained from every subject before enrolment in the study. Promastigotes were used to prepare clonal populations by serial dilution (Bhattacharyya et al., 2002) and five to eight clonal populations were prepared for each isolate. One clonal population from each of the isolates was used in the study. The details and history of these isolates are shown in Table 1. SSG-sensitive and -resistant strains MHOM/IN/83/AG83 and MHOM/IN/2005/BHU138, respectively, were maintained in golden Syrian hamsters (*M. auratus*) as previously described (Mukhopadhyay et al., 1999) and were used as references in the study. In order to allow comparisons of drug susceptibility results with other laboratories, a second strain (recommended by the Kaladrug-R consortium (www.leishrisk.net/kaladrug) and Sb-S) MHOM/NP/03/BPK206/0 clone 10 (Rijal et al., 2007) was used. Previous reports suggest that the activity index (AI) of BPK 206/0 against a WHO reference strain MHOM/ET/67/HU3 is one (Decuypere et al., 2005; Rijal et al., 2007).

All of the promastigotes were maintained in M-199 complete medium and late stage promastigotes with a starting inoculum of 1×10^5 parasites/ml were used for all of the experiments, including infection, unless mentioned otherwise. The host cells were infected with promastigotes at a ratio of 1:10 (Basu et al., 2006). After incubating cultures overnight or for 4 h at 37 °C and under 5% CO_2, non-phagocytosed promastigotes were washed off with serum free medium RPMI 1640 and treatment provided as described.

2.4. Preparation of drug stocks and drug assays

Sodium stibogluconate (a kind gift from Albert David, Kolkata, India) solution was prepared at 1 mg/ml in PBS, with dissolution of the powder being aided by incubation at 37 °C overnight, followed by sterile filtration. The drug solution appeared clear. The drug was serially diluted over six concentrations in RPMI 1640 medium supplemented with 10% FBS/100 IU/ml of penicillin/100 μg/ml of streptomycin in triplicate at each concentration. Stock solutions and dilutions were freshly prepared for each use. Infected MΦs were incubated with drug dilutions for a total of 48 h at 37 °C and under 5% CO_2. Untreated MΦs received RPMI complete medium and infection was determined 48 h post treatment. In summary, MΦs were infected for 24 h followed by drug treatment for another 48 h.

2.5. Evaluation of drug AIs

At endpoints, the coverslips were washed with PBS, dried, fixed with 100% methanol (Merck), stained with 10% Giemsa (SIGMA) and examined microscopically. One hundred MΦs/coverslip were scored and the amastigotes were enumerated (Basu et al., 2006). The average of three untreated cultures was taken as 100% control against which the percentage inhibition of infected MΦs in treated cultures was calculated. Half maximal effective concentration (EC_{50}) values for each of the isolates were estimated against each drug as described (Inocêncio da Luz et al., 2009; Kremb et al., 2010). The results were also expressed as AIs, defined previously (Rijal et al., 2007) for comparing results from different experiments: EC_{50} tested strain/EC_{50} reference strain (here BPK 206/0 clone 10).

2.6. RNA isolation, cDNA synthesis and real-time quantitative PCR (RT-qPCR) analysis of gene expression

2.6.1. RNA isolation and cDNA

All parasites were used for RNA extraction. They were immediately resuspended upon harvest and disrupted in Trizol solution (Invitrogen, California, USA). RNA was isolated according to the manufacturer's protocol, washed with 70% ethanol in diethyl pyrocarbonate (DEPC) water, and the quality and quantity were estimated in a Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The isolated and purified RNA was stored at −70 °C until further use. Total RNA (2 μg/reaction) was reverse transcribed at 37 °C with MMLV reverse transcriptase (Invitrogen) using conditions recommended by the manufacturer for a 15 mer oligo(dT).

Table 1
Clinical profiles of patients with visceral leishmaniasis from a hyperendemic zone of Bihar and from Bangladesh, India.

SL. No.	Isolate code	Patient's details		Zone	Treatment received
		Age (years)	Sex		
1.	MHOM/IN/09/BHU568/0	30	F	Muzaffarpur (Bihar)	Amphotericin B
2.	MHOM/IN/10/BHU796/1	18	F	Do	Miltefosine
3.	MHOM/IN/10/BHU814/1	29	F	Do	Miltefosine
4.	MHOM/IN/10/BHU872/6	18	M	Do	Amphotericin B
5.	MHOM/IN/09/BHU592/0	12	F	Do	Amphotericin B
6.	MHOM/IN/10/BHU782/0	15	M	Do	Miltefosine
7.	MHOM/IN/05/BHU138	6	na	Uttar Pradesh	Amphotericin B
8.	MHOM/IN/09BHU 764/0	12	M	Bihar	Miltefosine
9.	MHOM/IN/09/BHU744/0	30	M	Do	Miltefosine
10.	MHOM/IN/09/BHU573/0	10	M	Do	Paromomycin + miltefosine
11.	MHOM/IN/09/BHU575/0	60	M	Do	Amphotericin B
12.	MHOM/IN/09/BHU569/0	14	M	Do	Paromomycin + miltefosine
13.	MHOM/IN/09/BHU574/0	13	F	Do	Paromomycin
14.	MHOM/IN/09/BHU770/0	12	F	Do	Miltefosine
15.	MHOM/IN/09/BHU572/0	28	M	Do	AmBisome + miltefosine
16.	MHOM/BN/07/PG3	17	M	Bangladesh	SAG
17.	MHOM/BN/07/PG4	8	F	Do	SAG
18.	MHOM/IN/09/BHU581/0	16	M	Bihar	Paromomycin + miltefosine
19.	MHOM/BN/06/PG2	39	M	Bangladesh	SAG
20.	MHOM/NP/03/BPK206/0			Sunsari (Nepal)	SAG
21.	MHOM/IN/10/BHU816/1	12	F	Bihar	Miltefosine
22.	MHOM/IN/09/BHU777/0	15	F	Do	Miltefosine
23.	MHOM/IN/83/AG83	na	M	Unknown[a]	SAG
24.	MHOM/IN/09/BHU741/1	55	M	Bihar	Miltefosine

na, unknown and SL, serial No.
[a] Treated at The Calcutta School of Tropical Medicine.

2.6.2. RT-qPCR to estimate expression of putative genes responsible for SSG unresponsiveness

The cDNA synthesis and RT-qPCR were done as described elsewhere (Decuypere et al., 2005). Briefly, the resulting cDNA from the parasite RNA was diluted 7.5 times and subjected to qPCRs for expression profiling of eight genes coding for proteins involved in Sb(V) metabolism, referred to as target genes and one gene α-Tubulin referred to as the control gene. The qPCR contained 2X Sybr Green Supermix (Applied Biosystems, CA, USA) diluted twice, and forward and reverse primers as specified (Decuypere et al., 2005). The sequences of the forward and reverse primer are presented in Supplementary Table S1. Reactions were run on an Applied Biosystems 7500 Fast Real-Time PCR system using the following thermal profile: initial denaturation at 95 °C for 5 min followed by 30 cycles with denaturation at 95 °C for 30 s, annealing at 60 °C for 15 s and extension at 72 °C for 15 s. The PCR was immediately followed by a melt curve analysis using temperature increments of 0.5 °C every 30 s to ascertain whether the expected product was amplified and to ensure that no non-specific products or primer dimers (which could bias the quantification) were formed. Experiments on negative controls of cDNA synthesis (i.e., without reverse transcriptase) and no-template controls (i.e., without cDNA template) were also done for each gene. All reactions were done in duplicate, and their arithmetic average threshold cycle (C_t) was used for data analysis. The fold of gene expression compared with the control was calculated using the formula: Fold of expression = $2^{-\delta\delta C_t}$ by 7500 Fast System SDS software, version 1.4 (Applied Biosystems, CA, USA).

2.7. Determination of intracellular non-protein thiol in clonal populations

Stationary phase parasites, 2×10^6 each, were used for the study. Each sample was washed with sterile PBS and incubated with 5 μM of 5-chloromethylfluorescein-diacetate (CMFDA; Molecular Probes, CA, USA) for 15 min at 35 °C in PBS in the dark as described elsewhere (Sarkar et al., 2009). Flow cytometry was performed in a BDFACSAriaII cell sorter. Results were analysed using FACSDIVA software (BD Biosciences, San Jose, CA, USA).

2.8. Determination of membrane fluidity of parasite membrane

2.8.1. Fluorescence anisotropy (FA)

The membrane fluorescence and lipid fluidity of cells were measured using the method of Shinitzky and Inbar (1974) with slight modifications. Briefly, the fluorescent probe diphenylhexatriene (DPH) was dissolved in tetrahydrofuran at a 2 mM concentration. Two millimolar DPH solution was added to 10 ml of rapidly stirring PBS (pH 7.2). For labelling, 2×10^6 cells were mixed with an equal volume of DPH in PBS (Cf 1 μM) and incubated for 2 h at 37 °C. Thereafter the cells were washed thrice and resuspended in PBS. The cells were then fixed with 2% paraformaldehyde for 15 min. The cells were washed twice again and resuspended in 2 ml of PBS. The DPH probe bound to the membrane of the cell was excited at 365 nm and emission was recorded at 430 nm in a spectrofluorimeter. For trimethylamino-DPH (TMA-DPH), the cell suspension was incubated for 30 min at 1 μM TMA-DPH in PBS and the rest of the procedure was as for DPH. The TMA-DPH probe bound to the membrane of the cell was excited at 360 nm and emission was recorded at 430 nm in a spectrofluorimeter (Toplak et al., 1990). The FA value was calculated using the equation: $FA = (I_{II} - I_{\perp})/(I_{II} + 2I_{\perp})$, where I_{II} and $I_{\perp}$ are the fluorescent intensities oriented, respectively, parallel and perpendicular to the direction of polarisation of the excited light (Shinitzky and Inbar, 1974).

2.8.2. Extrinsic fluorescence quenching

The fluorescence intensity of 1-pyrene butyric acid (PBA) which partly partitions in water (Deumie et al., 1995) was measured by probing over the parasite cell surface. A dilution, 1 μM suspension of 1-PBA (1 mM stock in DMSO) was prepared in PBS (pH 7.2). Promastigotes (2×10^6) were washed twice with PBS. After incubating for 2 h at room temperature, the washed promastigotes were washed twice and finally resuspended in PBS. The fluorescence intensity of the membrane bound 1-PBA was analysed at

an excitation wavelength of 340 nm and emission wavelength of 380 nm in a spectrofluorimeter (Perkin Elmer LS55) using a 2 ml quartz cuvette. The fluorescence displayed by parasite membrane-bound 1-PBA was quenched independently in the presence of different concentrations (0.2, 2, and 20 mM) of quenchers, 2-Iodobenzoic acid (2-IBA) and Potassium Iodide (KI). Chemical structures, properties and excitation/emission wavelengths of the fluorescent probes and quenchers used in the study are presented in Supplementary Table S2. The fluorescence of the buffer and the different concentrations of quenchers were also measured and subtracted from the corresponding labelled cell suspension fluorescence. The fluorescence intensities at different concentrations were then optimised by dividing with unquenched fluorescence, plotted against the respective concentrations and a straight line equation was taken by best fit curve method (Xing et al., 1995). The slope for each curve was calculated and compared.

2.9. Quantification of surface sugar N-acetyl-D-galactosaminyl residue

Stationary phase parasites, 2×10^6 each, were used for the study. Each of the samples was washed with sterile PBS and incubated with FITC-conjugated lectins specific for a wide array of sugars at a dilution of 1:50 for 30 min in FACS buffer (PBS + 5% FBS). All of the lectins were in powdered form and were dissolved in sterile water as indicated in the manufacturer's protocol. The dilution factor was determined by measuring the fluorescence intensity of a range of dilutions in a spectrofluorimeter (Perkin Elmer LS55). The dilution which showed optimum fluorescence was used. The FITC-labelled promastigotes were subjected to flow cytometry analysis in a BDFACSAria II cell sorter. The mean fluorescence intensity was analysed by FACSDIVA software and accordingly the relative expression of surface sugar was determined.

2.10. Estimation of IL-10 in the culture supernatant

The MΦs were infected for 24 h and resulting culture supernatant was harvested. The cytokine IL-10 was assayed in the supernatant using an ELISA kit (BD Biosciences, CA, USA) according to the manufacturer's protocol.

2.11. Analysis of the expression of ABC transporters MRP1 and P-gp (MDR1)

The expressions of MRP1 and P-gp on the surface of the MΦ were determined by immunostaining, followed by flow cytometry (Basu et al., 2008). Briefly, the cells were stained with a primary anti-rabbit MDR1 and MRP1 antibody (Santa Cruz, CA, USA) followed by a secondary anti-rabbit whole IgG antibody conjugated with FITC (SIGMA). The appropriate isotype control was used in each case. Samples were fixed with 2% paraformaldehyde and the data was acquired using a BDFACSAria II cell sorter. The results were analysed by the FACSDIVA software (BD Biosciences, San Jose, CA, USA) (Basu et al., 2008).

2.12. Statistical analyses

Each experiment was performed three to five times and the results are expressed as mean ± S.D., or a Student's *t* test for significance was performed using Graphpad prism software, and a *P* value of <0.05 was considered to be significant. To find a correlation between the EC_{50} and other parameters, the Spearman rank correlation coefficient was used and expressed as *r*.

3. Results

3.1. Sensitivity of intracellular amastigotes to anti-leishmanial drugs

The clonal populations of the field isolates were subjected to tests for susceptibility to SSG on intracellular amastigotes (Basu et al., 2008). Infected MΦ ranged from 89% to 98%, and the number of amastigotes/100 MΦ ranged from 200 to 290. EC_{50} values ranged from 0.4 ± 0.3 to 30.3 ± 3.4 μg/ml, with a mean EC_{50} of 15.4 ± 1.85 μg/ml (Fig. 1). The isolates were grouped into two distinct categories based on their EC_{50} values. The results were also expressed in terms of AI using BPK 206/0 as a reference strain. Those isolates with an AI ⩾ 3 are considered to be as Sb-R, and an AI < 3 is considered as Sb-S (Rijal et al., 2007) (Table 2).

3.2. Gene expression profiling

The expressions of a series of genes that are responsible for thiol metabolism and are related to SSG transport were studied. Compared with Sb-R, all of the Sb-S isolates showed approximately 7.5 times over-expression of Aquaglyceroporin (AQP1) ($P < 0.05$, $r = -0.6$). The Sb-R isolates showed overall higher expression of MRPA ($P < 0.05$, $r = 0.7$, 25-fold) compared with the Sb-S isolates (Fig. 1A). We also observed that thiol dependent reductase (TDR) ($P < 0.05$, $r = 0.4$, 26-fold), γ-glutamylcysteine synthase (γ-GCS) ($P < 0.05$, $r = 0.6$, 4.5-fold), trypanothione reductase (TR) ($P < 0.05$, $r = 0.6$, 6-fold), ornithine decarboxylase (ODC) ($P < 0.05$, $r = 0.4$, 43-fold) and mercaptopyruvate sulfurtransferase (MST) ($P < 0.05$, $r = 0.5$, 5.5-fold) were significantly over-expressed in Sb-R compared with the Sb-S isolates (Fig. 1B). However, there is no strong correlation between the EC_{50} values and the expression levels of some thiol metabolising genes (Fig. 1), and we can see an obvious negative correlation between EC_{50} and AQP1 expression. Expression of cystathionine-β-synthase (CBS) did not show any significant difference between the two types of isolates, although on an average Sb-R showed 1.8-fold higher expression. The efficiency of PCR was above 90% and there was no amplification of primer dimers or non-specific product.

3.3. Intracellular thiol content of resistant and sensitive clonal populations

The intracellular thiol content of eight isolates from the Sb-R group and four from the Sb-S group was determined. It was observed that Sb-R had 1.5–6 times more intracellular non-protein thiol than the Sb-S isolates ($P < 0.05$) (Fig. 2). There is a high correlation ($r = 0.88$) between intracellular thiol content and SSG-unresponsiveness.

3.4. Determination of membrane fluidity in terms of FA and extrinsic fluorescence quenching

The membrane fluidity of *L. donovani* isolates was studied in terms of FA and extrinsic fluorescence quenching using appropriate fluorescence probes.

3.4.1. Anisotropy

It was observed that the Sb-R strain possesses relatively low FA compared with the Sb-S isolates when DPH was used as a probe, suggesting that the Sb-S membranes are significantly less fluid ($P < 0.05$) compared with Sb-R (Fig. 3). Similar results were observed with TMA-DPH.

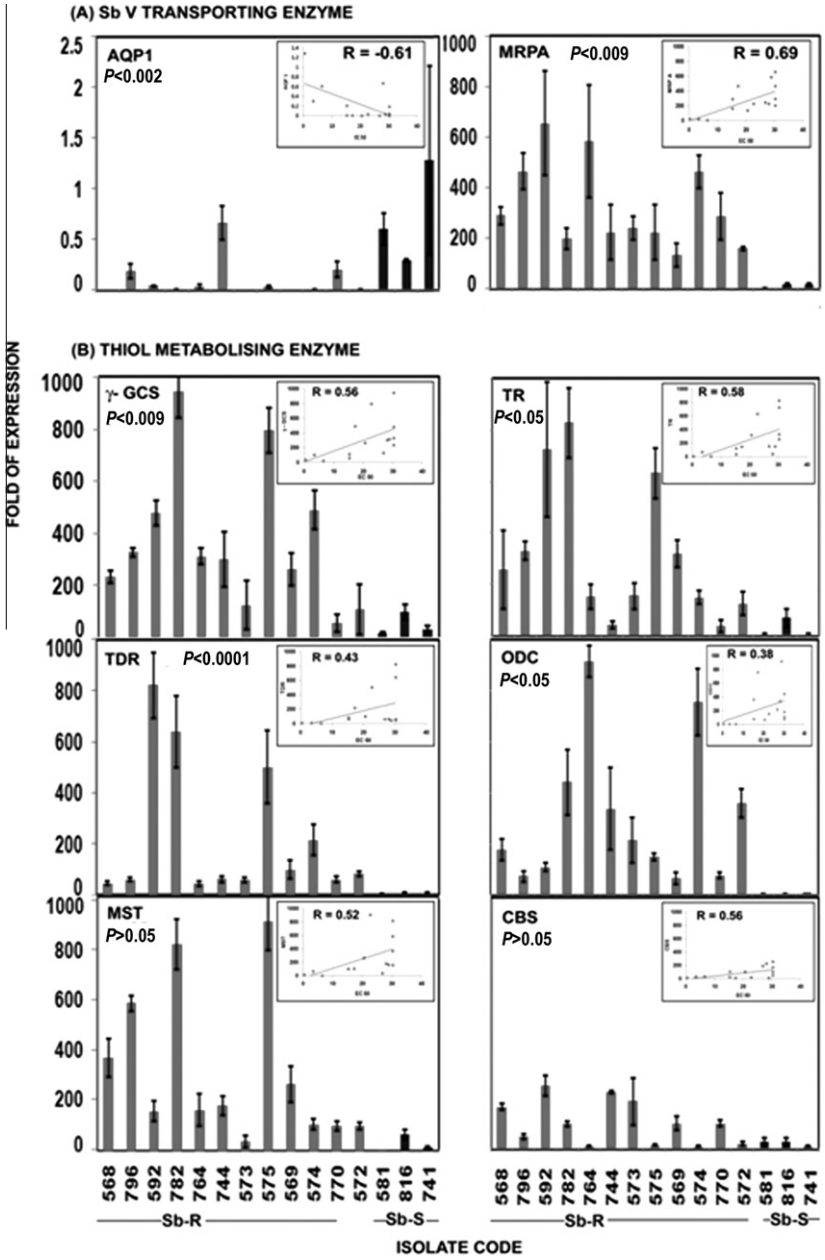

Fig. 1. Relative gene expression levels of target genes for antimony transport and thiol metabolism. (A) Aquaglyceroporin 1 (AQP1) and Multidrug Resistant Protein A (MRPA), (B) thiol dependent reductase (TDR), trypanothione reductase (TR), γ-glutamylcysteine synthase (γ-GCS), ornithine decarboxylase (ODC), cystathionine-β-synthase (CBS) and Mercaptopyruvate Sulphur Transferase (MST) of the Sb-S isolates (BHU 581, 741, 816) and Sb-R isolates (BHU 568, 569, 572, 573, 574, 575, 592, 764, 782 and 796) of *Leishmania donovani*. Data are expressed as the mean ± S.D. of three independent experiments. Each scatter plot in the inset represents the correlation between the EC_{50} of the isolates against sodium stibogluconate (SSG) and the respective gene expression.

3.4.2. Quenching

The quencher 2-IBA was used due to its hydrophobic nature, resulting in the partitioning in the hydrophobic region of the cell. The other quencher used, KI, is ionic and remains in hydrated form in an aqueous environment (Deumie et al., 1995). Fluorescence quenching was found to be significantly higher (for both 2-IBA and KI) in the Sb-R isolates ($P < 0.0001$) compared with the Sb-S ones. When 2-IBA was used as a quencher at a 20 mM concentration, the relative fluorescence quenching was higher and fluorescence of 1-PBA totally ceased. The extent of quenching was compared in terms of the slope (m) of each straight line equation (Fig. 4). The data showed that the slope (m) is higher in the case of Sb-R compared with Sb-S.

3.5. Detection of *N*-acetyl-D-galactosaminyl residue on the surface of promastigote membrane

We analysed whether there is any relationship between drug susceptibility and surface glycoconjugate expression in the parasites. FITC-labelled horsegram (*Dolichos biflorus*) and coral tree (*Erythrina crystagalli*) lectins were used as probes to study the presence of surface sugar *N*-acetyl-D-galactosaminyl residues in promastigotes by flow cytometry (Hamelryck et al., 1999). It was revealed that Sb-R isolates, on average, show a significantly higher expression of *N*-acetyl-D-galactosaminyl ($P < 0.05$, 6-fold) compared with the Sb-S isolates. On the other hand, binding of the mannose-specific lectin FITC-Con A was comparable between

Table 2
Susceptibility of intracellular amastigotes of *Leishmania donovani* isolates to sodium stibogluconate (SSG), represented by EC_{50} values and activity indices against control strain BPK 206/0 cl 10.

SL. No.	Isolate code	In absence of SbV		EC_{50} (SbV μg/ml)	AI against BPK 206/0
		% Infected MΦ	Number of amastigotes/100 MΦ		
1	BHU 568	93	286	30.3 ± 3.4	6.50
2	BHU 796	89	252	30.3 ± 3.9	6.50
3	BHU 814	96	231	30.3 ± 7.2	6.50
4	BHU 872	92	241	30.3 ± 7.4	6.50
5	BHU 592	98	297	30.3 ± 4.5	6.50
6	BHU 782	92	250	30.3 ± 4.3	6.50
7	BHU 138	89	242	29.2 ± 2.5	6.23
8	BHU 764	91	239	28.86 ± 4.4	6.19
9	BHU 744	93	234	28.06 ± 1.8	6.02
10	BHU 573	97	255	26.82 ± 4.1	5.75
11	BHU 575	92	245	22.73 ± 3.7	4.87
12	BHU 569	94	263	20.60 ± 5.6	4.42
13	BHU 574	97	285	17.27 ± 6.2	3.70
14	BHU 770	98	201	15.32 ± 3.6	3.28
15	BHU 572	96	276	15.30 ± 5.9	3.28
16	PG3	88	224	8.03 ± 3.6	1.72
17	PG4	86	242	7.27 ± 2.1	1.56
18	BHU 581	91	208	6.51 ± 1.5	1.39
19	PG2	87	242	5.75 ± 0.9	1.23
20	BPK 206/0	88	222	4.66 ± 1.1	1.00
21	BHU 816	89	245	3.44 ± 2.2	0.73
22	BHU 777	84	241	3.32 ± 2.1	0.71
23	Ag83	87	221	1.52 ± 1.2	0.32
24	BHU 741	86	288	0.44 ± 0.3	0.09

Serial numbers in bold and regular denote unresponsive (Sb-R) and sensitive (Sb-S) to SbV, respectively. The percentage of infected MΦs and the number of amastigotes/100 MΦs at 72 h in the absence of drug were enumerated. Results are given as mean ± S.D.
SbV, pentavalent antimony; AI, activity index and SL, serial number.

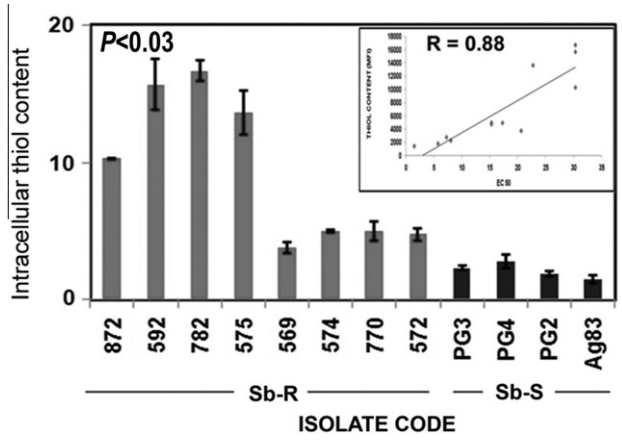

Fig. 2. Thiol content in antimony-resistant (Sb-R) and antimony-sensitive (Sb-S) isolates of *Leishmania donovani*. Thiol content was measured using a fluorescence probe 5-chloromethylfluorescein-diacetate (CMFDA) and presented in terms of mean fluorescence intensity (MFI) values. Cells (2×10^6) were incubated in 5 μM of CMFDA in PBS at 37 °C for 15 min. Data are expressed as the mean MFI ± S.D. of three independent experiments. Inset shows the scatter plot representing correlation between EC_{50} values against sodium stibogluconate (SSG) and the intracellular thiol content of each isolate.

Sb-S and Sb-R isolates (Fig. 5). There is a high correlation ($r = 0.76$) between the expression of terminal glycoconjugates with SSG-unresponsiveness, indicating that drug resistance results in over-expression of *N*-acetyl-D-galactosamine residues.

3.6. Differential induction of the proinflammatory cytokine IL-10 in MΦs infected with isolates differing in SSG sensitivity

Previously, we have shown that infection of bone marrow DCs with Sb-R induces higher IL-10 production compared with Sb-S isolates (Haldar et al., 2010). It was important to determine whether this also holds true for MΦs, as these are regarded as primary targets for *Leishmania* infection. Murine MΦs were therefore infected with different isolates and levels of IL-10 were measured in the culture supernatant. It was observed that IL-10 production was found to be 3–4-fold higher in the case of MΦs infected with Sb-R compared with Sb-S (Fig. 6). There was a high correlation ($r = 0.88$) between SSG resistance of the isolates and IL-10 generation by infected host cells.

3.7. Expression of cell surface ABC transporter proteins, MDR1 (P-gp) and MRP1

Over-expression of ABC transporters such as MDR1 (P-gp) or MRP1 on host cells causes multiple drug resistance in different diseases (Borst and Elferink, 2002) such as cancer (Gottesman et al., 2002; Luqmani, 2005), HIV infection or mycobacterium-HIV co-infection (Gollapudi et al., 1994). It has been reported that these ABC transporters are over-expressed in leishmaniasis (Basu et al., 2008). We analysed the expression status of MRP1 and P-gp on the surface of the MΦs infected with these isolates. We found that Sb-R induces on average five times over-expression of both transporters in MΦs compared with the Sb-S isolates ($P < 0.05$) (Fig. 7). This results very clearly showed that the degree of SSG unresponsiveness in *L. donovani* isolates has a direct impact on the ABC transporter expression in the infected host cell ($r = 0.94$).

4. Discussion

It is clear that 78% of recent isolates from the hyperendemic zone of Bihar showed different degrees of refractoriness to SSG while the remaining ones showed sensitivity towards SSG-mediated intracellular clearance (Table 2). There are reports of increased thiol content in the Sb-R than in the Sb-S isolates (Mukhopadhyay et al., 1996) and our data support such a notion. The high correlation ($r = 0.88$) between SSG resistance and

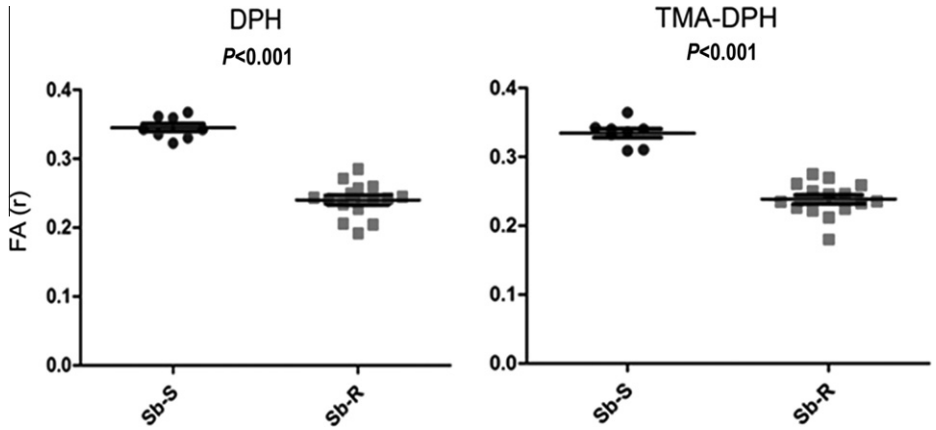

Fig. 3. Measurement of fluorescence anisotropy (FA) of the promastigotes of *Leishmania donovani*. The FA value was measured using diphenylhexatriene (DPH) and trimethylamino-diphenylhexatriene (TMA-DPH) as probes. The fluorophore was excited at 365 or 360 nm, emission intensity was recorded at 430 or 435 nm for DPH and TMA-DPH, respectively, and the FA was calculated. Data represent the comparison of FA values between antimony-resistant (Sb-R) and antimony-sensitive (Sb-S) isolates.

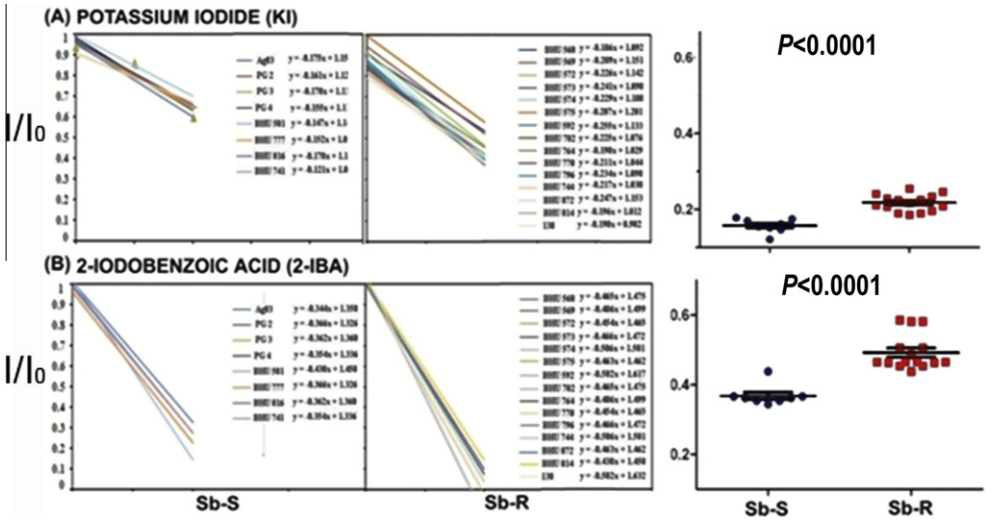

Fig. 4. Fluorescence quenching of membrane bound probes in promastigotes of *Leishmania donovani*. Quenching of membrane bound 1-pyrene butyric acid (1-PBA) with (A) Potassium Iodide (KI) and (B) 2-Iodo Benzoic Acid (2-IBA) was performed. The fluorophore was excited at 340 nm and emission intensity was recorded at 380 nm. Data are represented by plotting the ratio of quenched and unquenched intensities (I/I_0) versus concentration of the quencher, in the form of a straight-line equation to obtain best-fit line. Comparison of slope values between antimony-resistant (Sb-R) and antimony-sensitive (Sb-S) isolates and their median values are represented for both of the quenchers.

intracellular non-protein thiol content in the Indian field isolates indicates that there may be an association between these two observations. This led us to probe the possible relationship between the intracellular thiol content and the status of gene expression of thiol metabolising enzymes (Fig. 1). In *Leishmania* parasites, the bis-glutathionyl spermidine conjugate trypanothione is the major source of thiol (Fairlamb et al., 1985). Over-expression of ODC and γ-GCS in resistant isolates aids the production of glutathione and spermidine, which are precursors for the reduced trypanothione (Grondin et al., 1997; Haimeur et al., 1999). Our results show over-expression of some thiol metabolising enzymes such as ODC, GCS, TDR and TR in Sb-R isolates, in contrast with a previous report (Decuypere et al., 2005). However, the present report examines different isolates than those used by Decuypere et al. (2005) and recent findings showed that gene expression profiles can vary strongly between Sb(V)-resistant isolates (Adaui et al., 2011a,b) which are in agreement with our report. Over-expression of CBS aids in the production of cysteine, the other thiol source of trypanosomatids (Nozaki et al., 2001). The reduced thiol trypanothione forms a complex with potentially toxic Sb(III) and renders them inactive by transportation through MRPA into the parasite vacuole (Légaré et al., 2001). Our study also shows that AQP1 expression is higher in Sb-S isolates compared with Sb-R ones. AQP1 helps Sb(III) uptake into the cell (Gourbal et al., 2004; Marquis et al., 2005; Maharjan et al., 2008).

Drug resistance induces modifications in the cell surface of the parasite (Vanaerschot et al., 2010) as well as in membrane lipid composition (t'Kindt et al., 2010). Several reports indicate an increase in the membrane fluidity in *L. donovani* isolates with amphotericin B resistance (Mbongo et al., 1998), whereas the case is reversed for miltefosine resistance (Rakotomanga et al., 2005). In the present study, membrane anchor probes DPH and TMA-DPH were used to assess membrane fluidity of *L. donovani* isolates. Both of the probes readily partition from aqueous dispersions into membranes and other lipid assemblies, accompanied by strong fluorescence. The lipid–water partition coefficient (K_p) for TMA-DPH is lower than for DPH, reflecting the increased water solubility caused by the polar substituents (Huang and Haugland, 1991). The fluorescence decay lifetime of TMA-DPH is more sensitive to changes in lipid composition and temperature than is the

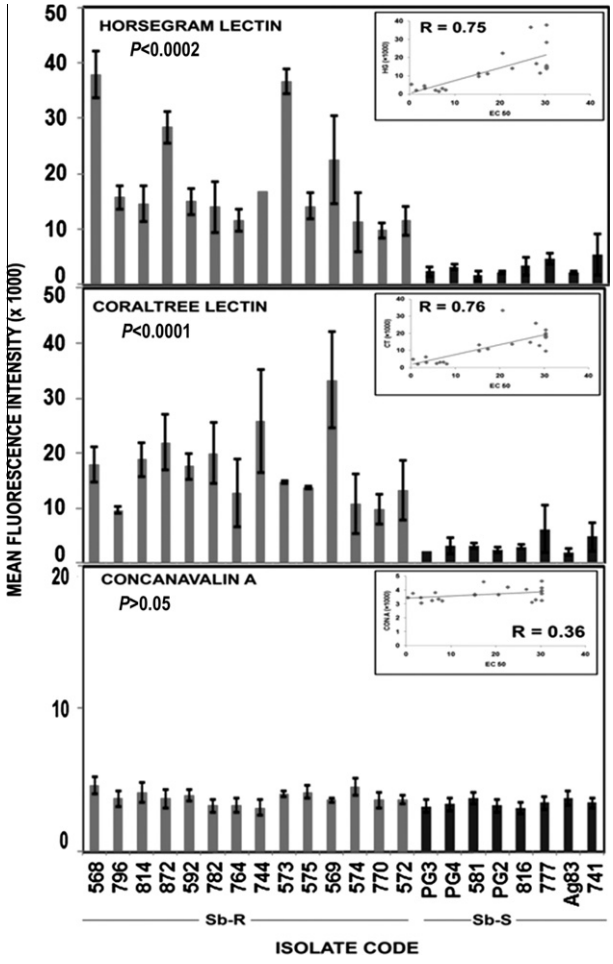

Fig. 5. Flow cytometric analysis of differential expressions of terminal *N*-acetyl-D-galactosaminyl residue in promastigotes of *Leishmania donovani*. The plot shows the binding of FITC-labelled horsegram (*Dolichos biflorus*), coral tree (*Erythrina crystagalli*) and mannose-specific lectins, Concanavalin A in antimony-resistant (Sb-R) and antimony-sensitive (Sb-S) isolates. Each scatter plot in the inset represents the correlation between the EC_{50} of the isolates with the presence/absence of surface sugar residues.

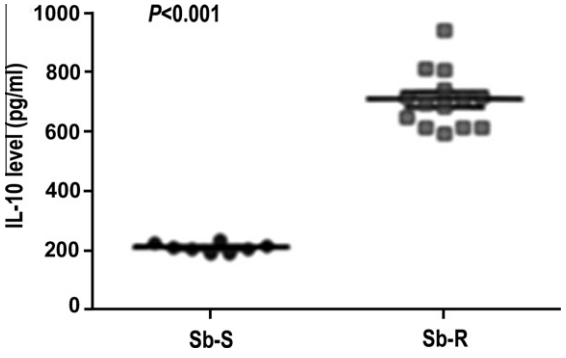

Fig. 6. Analysis of IL-10 expression from macrophages (MΦs) infected either with antimony-sensitive (Sb-S) or antimony-resistant (Sb-R) isolates of *Leishmania donovani*. MΦs were infected either with Sb-S or Sb-R isolates for 24 h or left uninfected. The culture supernatants were harvested to determine IL-10 content by ELISA. The data are represented as a dot plot showing the median values of IL-10 (pg/ml).

fluorescence decay lifetime of DPH (Zolese et al., 1990). Here we show that Sb-S shows relatively higher FA compared with Sb-R (Fig. 3), suggesting the increased fluidity of the latter as membrane fluidity is inversely proportional to FA (Lentz, 1989; Lakowicz, 2006). It has been reported that the proportion of phosphatidylcholine with high fatty acyl unsaturation is higher for the Sb-R isolates compared with Sb-S (t'Kindt et al., 2010). As the unsaturation of the fatty acyl chain increases, the packing efficiency of the lipids decreases due to kink formation (Subczynski and Wisniewska, 2000).

The increased fluidity in Sb-R also received support from fluorescence quenching studies. Quenching imparted by 1-PBA was studied using two distinct classes of probes, 2-IBA and KI. 2-IBA is a hydrophobic probe and might readily interact with the lipid environment of the membrane, whereas KI is hydrated and might have limited access to the hydrophobic surface (Shinitzky and Barenholz, 1978). When the extent of quenching, I/I_0, was plotted as a function of quencher concentration and the resulting slope value was compared between Sb-S and Sb-R with respect to each quencher, Sb-R isolates showed much higher quenching by both 2-IBA and KI. This observation reinforces the FA data, suggesting that Sb-R parasites are more fluid than Sb-S ones. Based on the results with external quenchers it is tempting to speculate that Sb-S and Sb-R might react differently with hydrophobic anti-leishmanial drugs.

Glycoconjugates play some role in parasite-host interactions, especially in the mammalian stage (Descoteaux and Turco, 1999; Mukhopadhyay and Mandal, 2006). They might also serve as a ligand for the MΦ receptor and protect the parasite against complement-mediated lysis (Alexander and Russell, 1992). *Leishmania* also secrete a family of heavily glycosylated proteins and proteoglycans that are important for parasite virulence (Descoteaux and Turco, 1999). The metacyclogenesis in *L. donovani* is associated with a change in surface glycoconjugates (da Silva and Sacks, 1987; Descoteaux et al., 1995; Denny et al., 2001), reported to be higher in the resistant isolates (Vanaerschot et al., 2010). Our observations suggest that the Sb-R isolates expressed surface glycoconjugates with a terminal sugar *N*-acetylgalactosaminyl residue, which was almost absent in Sb-S (Fig. 5).

Our previous study demonstrated that Sb-R can regulate the maturation and activation of DCs and induce IL-10 production from DCs (Haldar et al., 2010). Our results also clearly showed that Sb-R but not Sb-S up-regulate IL-10 production from MΦ. When the MΦs were infected with either Sb-R or Sb-S isolates, at the end point there was no significant difference in the percentage of infected MΦs or number of amastigotes/100 MΦs. Vanaerschot et al. (2010) reported that metacyclogenesis is favoured in Sb-R parasites. It is tempting to speculate that the increased number of metacyclics in Sb-R than Sb-S might also contribute to IL-10 production from MΦ. It is already known from previous studies that IL-10, a potent suppressor of anti-leishmanial immunity, minimises responsiveness to SSG (Murphy et al., 2001; Murray et al., 2002) and the IL-10 level is much higher in sera of kala azar patients harbouring Sb-R parasites (Thakur et al., 2003). MRP1 and P-gp are the two types of transporter proteins that belong to the ABC transporter superfamily. ABC transporters are usually responsible for the efflux of drugs or other xenobiotics from the cell to prevent accumulation of any cytotoxic agent within the cell. It was previously reported that the Sb unresponsive isolates cause the MΦ to over-express the ABC transporters (Basu et al., 2008). These transporters efflux SSG out of the cell and a positive correlation is observed ($r = 0.94$) between the Sb unresponsiveness and expression levels of these transporters on the infected MΦ (Fig. 7).

Taken together, our study indicates that Sb-R isolates are very different from the Sb-S ones in a wide variety of biochemical and biophysical parameters as well as in their ability to interact with host cells. Although our scientific knowledge concerning drug resistance is in its infancy, understanding such a mechanism allows one to predict the outcome, permitting the optimised use of current drugs and introduction of new drugs. SSG has not been in use for some time on the Indian sub-continent, and although the removal

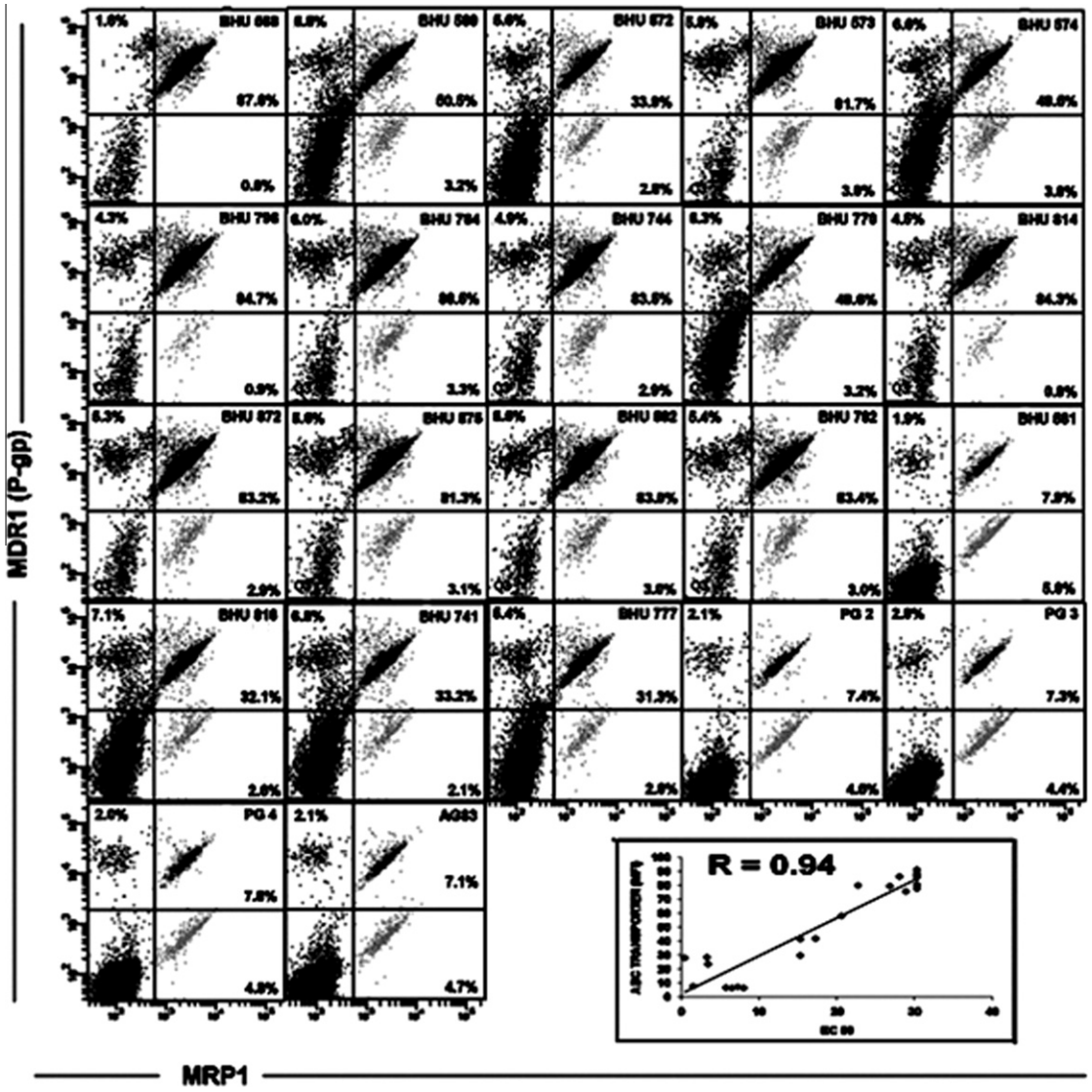

Fig. 7. Analysis of ATP Binding Cassette (ABC) transporter expression in macrophages (MΦs) by flow cytometry. MΦs were infected either with antimony-sensitive (Sb-S) or antimony-resistant (Sb-R) isolates of *Leishmania donovani* and Multidrug Resistant Protein 1 (MRP1) and P-glycoprotein (MDR1) expression were quantified by flow cytometry. Dot plots show percentages of double-positive populations of MΦs infected with either Sb-R or Sb-S isolates. The inset shows a scatter plot representing the correlation between the EC_{50} against sodium stibogluconate (SSG) and the ABC transporter induction in host cells by each of the isolates.

of drug pressure is expected to allow the return of sensitive organisms by natural selection, this is not universally accepted (Lipsitch and Levin, 1997a,b). Recent reports suggest that Sb-R isolates develop a series of molecular adaptations that lead to an increased fitness even in the absence of the drug. It was shown on one hand that the metacyclogenesis rate is higher in Sb-R and that they are also more infective at the early stages of infection (Vanaerschot et al., 2010). Moreover there are reports on the identification of a gene in *Leishmania infantum* that mediates resistance to Sb(III) (Choudhury et al., 2008). It might be possible that due to horizontal transfer of such genes in these isolates, their clonal expansion might be responsible for persistence of Sb resistance in the field even in the absence of the drug. At the moment, however, there is no simple tool to assess how this rampant SSG resistance in the field would influence the efficacy of newer versions of drugs currently in use.

Acknowledgements

This research was supported by the European Union Kaladrug-R Project (Health-F3-2008-222895) and the Council of Scientific and Industrial Research, New Delhi, India. RM, SM and BM are recipients of fellowships from the Council of Scientific and Industrial Research, New Delhi.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpara.2011.07.013.

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