

Inhibition of ABC Transporters Abolishes Antimony Resistance in *Leishmania* Infection[∇]

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The emergence of antimony (Sb) resistance has jeopardized the treatment of visceral leishmaniasis in various countries. Previous studies have considered the part played by leishmanial parasites in antimony resistance, but the involvement of host factors in the clinical scenario remained to be investigated. Here we show that unlike infection with Sb-sensitive (Sb^s) *Leishmania donovani*, infection with Sb-resistant (Sb^r) *L. donovani* induces the upregulation of multidrug resistance-associated protein 1 (MRP1) and permeability glycoprotein (P-gp) in host cells, resulting in a nonaccumulation of intracellular Sb following treatment with sodium antimony gluconate (SAG) favoring parasite replication. The inhibition of MRP1 and P-gp with resistance-modifying agents such as lovastatin allows Sb accumulation and parasite killing within macrophages and offers protection in an animal model in which infection with Sb^r *L. donovani* is otherwise lethal. The occurrence of a similar scenario in clinical cases is supported by the findings that unlike monocytes from SAG-sensitive kala-azar (KA) patients, monocytes from SAG-unresponsive KA patients overexpress P-gp and MRP1 and fail to accumulate Sb following in vitro SAG treatment unless pretreated with inhibitors of ABC transporters. Thus, the expression status of MRP1 and P-gp in blood monocytes may be used as a diagnostic marker for Sb resistance and the treatment strategy can be designed accordingly. Our results also indicate that lovastatin, which can inhibit both P-gp and MRP1, might be beneficial for reverting Sb resistance in leishmaniasis as well as drug resistance in other clinical situations, including cancer.

The emergence of antimony-resistant (Sb^r) visceral leishmaniasis (VL) in various parts of the world (1, 8, 17, 48) has severely compromised control of the disease. Among alternative drugs, pentamidine is toxic; amphotericin B is both expensive and toxic, with reported cases of resistance (8, 48); and oral miltefosine is limited by cost, contraindications, and emerging resistance (1, 8, 41, 53). Therefore, an understanding of the mode of resistance and an identification of cost-effective therapeutic combinations have become major issues.

ATP binding cassette (ABC) transporters have been widely reported to export xenobiotics (24, 55) and cause drug resistance in various diseases such as cancer (23). Earlier studies have reported the expression of analogs of ABC transporters on the surfaces of Sb^r strains of *Leishmania* promastigotes (27, 35, 41), believed to efflux antimonials. However, the demonstration of these transporters in promastigotes may not be very relevant to clinical situations. There are a few reports available on the expression of similar transporters in laboratory isolates

of in vitro-developed Sb^r strains of leishmanial amastigotes (15) or on amastigotes from field isolates of Sb^r *L. donovani* (10, 51). Although sodium antimony gluconate (SAG) kills leishmanial amastigotes directly at higher doses in vitro as reported previously (54), a much lower dose is required for killing the parasite within macrophages (Mφ) (25). Furthermore, SAG fails to act in immunocompromised hosts, such as patients who are suffering from AIDS or receiving immunosuppressive agents (19, 36) and nude (39) and severe combined immunodeficient mice (16). Several studies have shown that endogenous interleukin-2 (IL-2) (38), IL-4 (2, 40), and IL-12 (37) influence the effectiveness of chemotherapy with pentavalent antimony. Together, these findings are inclined to indicate the requirement of a somewhat functional immune system for SAG action. We also demonstrated earlier that SAG induces the Mφ to produce leishmanicidal molecules like nitric oxide (NO) and reactive oxygen species (ROS), leading to the elimination of intracellular *L. donovani* (33). Thus, SAG may act both directly and through activation of the host's Mφ. Moreover, SAG can also induce the generation of gamma interferon from splenic lymphocytes, the proliferation of splenocytes (34, 44), and even the proliferation of IL-2-dependent CTLL2 and HT2 T-cell lines in the absence of IL-2 (34) and can upregulate NF-κB activation and the expression of major histocompatibility complex I in normal as well as *L. donovani*-infected Mφ (J. Mookerjee Basu, P. Sen, and S.

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TABLE 1. Profiles of the confirmed SAG-responsive/-unresponsive KA patients^a

Patient no.	Residence area in Bihar (district)	Age (yr)	Sex	Splenic parasites ^b	Previous therapy	Remarks
1	Vaisali	24	M	3+	SAG, full course ^d	SAG unresponsive
2	Nalanda	35	F	2+	SAG, full course ^d	SAG unresponsive
3	Patna	45	F	3+	SAG, full course ^d	SAG unresponsive
4	Patna	35	F	2+	SAG, full course ^d	SAG unresponsive
5	Motihari	52	F	2+	SAG, full course ^d	SAG unresponsive
6	Vaisali	26	M	3+	SAG, full course ^d	SAG unresponsive
7	Vaisali	19	F	3+	SAG, full course ^d	SAG unresponsive
8	Nalanda	29	M	2+	SAG, full course ^d	SAG unresponsive
9	Nalanda	34	M	2+	SAG, full course ^d	SAG unresponsive
10	Vaisali	42	F	4+	SAG, full course ^d	SAG unresponsive
11	Nalanda	32	M	3+ (before treatment)	Received SAG for 3 wk	Clinically responsive to SAG ^c
12	Gopalgung	50	F	2+ (before treatment)	Received SAG for 25 days	Clinically responsive to SAG ^c
13	Siwan	45	F	3+ (before treatment)	Received SAG for 18 days	Clinically responsive to SAG ^c
14	Siwan	35	M	2+ (before treatment)	Received SAG for 22 days	Clinically responsive to SAG ^c
15	Gopalgung	25	M	4+ (before treatment)	Received SAG for 17 days	Clinically responsive to SAG ^c
16	Gopalgung	25	M	2+ (before treatment)	Received SAG for 25 days	Clinically responsive to SAG ^c
17	Gopalgung	25	M	3+ (before treatment)	Received SAG for 27 days	Clinically responsive to SAG ^c
18	Gopalgung	25	M	2+ (before treatment)	Received SAG for 23 days	Clinically responsive to SAG ^c
19	Siwan	45	F	2+ (before treatment)	Received SAG for 24 days	Clinically responsive to SAG ^c
20	Siwan	45	F	2+ (before treatment)	Received SAG for 25 days	Clinically responsive to SAG ^c

^a F, female; M, male.

^b Determined microscopically from stained smears of splenic aspirates (7). Numbers are standard parasite burden grades determined by counting amastigotes versus host cells from Giemsa-stained slides.

^c Appearance of clinical symptoms of cure-like remission of fever, increase in hemoglobin level and white blood cell count and reduction in spleen size, ultimately checked for reduction of parasite in spleen after completion of treatment.

^d Received 20 mg/kg body weight of SAG for 28 days are indicated as full course of SAG.

Roy, unpublished observations), indicating that SAG may activate the immune system. Therefore, it is necessary to decipher the role played by the host cell, if any, in Sb unresponsiveness.

In this study, we show that infection with Sb^r *L. donovani* (but not Sb-sensitive [Sb^s] *L. donovani*) upregulates the host's ABC transporters and causes Sb nonretention and that host cells also play a role in the induction of resistance. We also propose a prognostic test for early identification of SAG-unresponsive cases. Moreover, we also identify a widely used antihypercholesteremic drug as a resistance-modifying agent (RMA) and propose a new strategy for the treatment of SAG-unresponsive kala-azar (KA) cases.

MATERIALS AND METHODS

Antibodies and other reagents. Anti-multidrug resistance-associated protein 1 (MRP1) and anti-MDR1 were obtained from Santa Cruz Biotech, Inc. Fetal bovine serum was obtained from Invitrogen Corporation (Carlsbad, CA). Inhibitors (lovastatin, verapamil, and probenecid), Giemsa stain, *N*-(1-naphthyl)-ethylenediamine dihydrochloride, sulfanilamide, starch, penicillin, streptomycin, medium 199, RPMI 1640, doxorubicin (Dox), and trypan blue were from Sigma (St. Louis), and 2',7'-dichlorodihydrofluorescein diacetate was from Molecular Probes (Eugene, OR). Preservative-free sodium stibogluconate/SAG was the kind gift of Albert David India Ltd. (Calcutta, India). SAG was diluted in RPMI medium 1640 (pH 7.4) just before being added to cultures.

Parasite strains. Three strains of *L. donovani*, SAG-sensitive (Sb^s) MHOM/IN/1983/AG83 (AG83) (45) and two SAG-resistant (Sb^r) strains, GE1F8R (raised in vivo in hamsters) (4) and K39 (isolated from a SAG-unresponsive patient) (46), were used. Amastigotes obtained from the spleens of infected hamsters were cultured at 22°C to obtain promastigotes (6).

Animals. BALB/c mice originally obtained from Jackson Laboratories, (Bar Harbor, ME) and golden Syrian hamsters (*Mesocricetus auratus*) originally obtained from the National Institute of Nutrition, Hyderabad, India, both reared in the institute's animal facilities, were used for experimental purposes, with prior approval of the animal ethics committee.

Cell lines and cell culture. Four different cell lines were used in this investigation, and they were (i) Dox-resistant MRP1-overexpressing Ehrlich ascites carcinoma (EAC/Dox) (32), (ii) EAC/S, a parental Dox-sensitive counterpart of EAC/Dox cells lacking overexpression of MRP1, (iii) Dox-resistant human acute T-lymphoblastic leukemia cell line CEM-ADR5000 overexpressing ABCB1/MDR1/permeability glycoprotein (P-gp) (14) (kind gift from T. Efferth, German

TABLE 2. Profiles of the untreated KA patients from SAG-responsive or -unresponsive zones^a

Patient no.	Residence area in Bihar (district)	Age (yr)	Sex	Splenic parasites ^b	Previous therapy	SAG zone
1	Gopalgung	35	F	3+	NT	R
2	Gopalgung	33	M	2+	NT	R
3	Gopalgung	24	F	2+	NT	R
4	Siwan	28	F	3+	NT	R
5	Siwan	40	M	2+	NT	R
6	Siwan	45	M	2+	NT	R
7	Gopalgung	43	M	3+	NT	R
8	Gopalgung	34	F	2+	NT	R
9	Siwan	28	F	2+	NT	R
10	Gopalgung	42	F	2+	NT	R
11	Vaisali	31	M	2+	NT	U
12	Vaisali	23	F	3+	NT	U
13	Vaisali	35	M	2+	NT	U
14	Vaisali	28	F	2+	NT	U
15	Vaisali	25	F	3+	NT	U
16	Vaisali	21	M	4+	NT	U
17	Samastipur	23	M	3+	NT	U
18	Vaisali	43	M	2+	NT	U
19	Vaisali	28	M	3+	NT	U
20	Vaisali	28	F	2+	NT	U

^a F, female; M, male; NT, no previous treatment; R, SAG-responsive zone; U, SAG-unresponsive zone.

^b Determined microscopically from stained smears of splenic aspirates (7). Numbers are standard parasite burden grades determined by counting amastigotes versus host cells from Giemsa-stained slides.

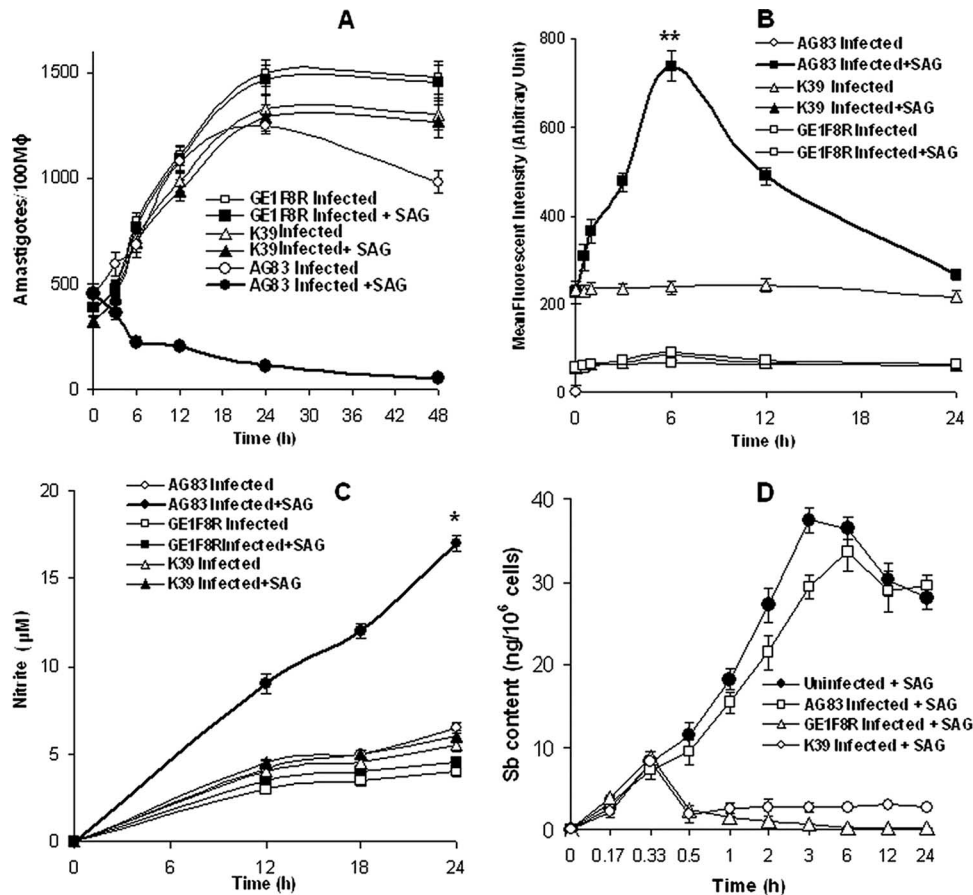


FIG. 1. Infection of Mφ with Sb^s *L. donovani* (AG83) and Sb^f *L. donovani* (K39/GE1F8R) differentially affect SAG-mediated elimination of intracellular parasites, the generation of ROS and NO, and the accumulation of Sb. Mφ from normal BALB/c were infected in vitro with *L. donovani* promastigotes and kept either untreated or treated with SAG (10 μg/ml). (A) Intracellular parasite number was determined for Giemsa-stained preparations. The replication of Sb^f *L. donovani* (but not Sb^s *L. donovani*) continued. Measurement of ROS (B) and NO (C) generations showed that SAG induced a significant (*, $P < 0.001$; **, $P < 0.005$) increase in ROS generation at 6 h and NO generation at 24 h in Sb^s-Mφ but not in Sb^f-Mφ. (D) Intracellular total Sb content was measured by atomic absorption spectroscopy; total Sb content was also measured in uninfected Mφ kept as controls following SAG treatment. Significant amounts of Sb accumulated in Sb^s-Mφ but not in Sb^f-Mφ ($P < 0.0001$) by 6 h posttreatment. Error bars indicate standard deviations.

Cancer Research Center, Heidelberg, Germany), and (iv) CCRF/CEM, a parental Dox-sensitive counterpart of CEM-ADR 5000, a human acute T-lymphoblastic leukemia cell line lacking overexpression of P-gp.

Peritoneal Mφ were obtained from BALB/c mice as described previously (33) and used for further experimentations. Splenic Mφ were isolated from BALB/c mice infected for 2 months with Sb^s or Sb^f *L. donovani* as described previously (45).

***L. donovani* infection (in vitro and in vivo) and determination of parasite burden.** Mφ were infected with either Sb^s or Sb^f *L. donovani* promastigotes as described previously (33). The number of Mφ infected with the intracellular *L. donovani* parasite was enumerated under Giemsa staining as described previously (33).

BALB/c mice (4 to 6 weeks old) were infected with 1×10^7 promastigotes (AG83, GE1F8R, or K39)/0.1 ml per animal via the intracardiac route (45), and mice infected for 2 months were used for experimental purposes.

Golden hamsters (4 weeks old) were infected with K39 (2×10^7 promastigotes/0.1 ml per animal) or GE1F8R (5×10^6 amastigotes/0.1 ml per animal) via the intracardiac route (on day 0) (4). For subsequent experiments, hamsters infected with GE1F8R for 30 days and hamsters infected with K39 for 60 days were used, because at these time points, the two groups of hamsters showed almost equal parasite burdens in spleens and livers. The parasite burden was determined by limiting the dilution of tissue samples (4).

Peripheral blood sample of patients. Excesses of blood drawn for routine examinations of confirmed KA patients, confirmed as either unresponsive to SAG (patients who reported to the clinic with KA within 2 months of receiving

full courses of SAG) or sensitive to SAG (KA patients who showed improvement of clinical symptoms of cure-like remissions of fever, increases in hemoglobin levels and white blood cell counts, and reductions in spleen size and splenic parasites after 3 weeks of SAG treatment), or from untreated (fresh) KA cases from zones of SAG resistance (SAG-resistant zone) or SAG sensitivity (SAG-sensitive zone) were collected as samples from the Department of Clinical Biochemistry, Hospital Unit, Rajendra Memorial Research Institute of Medical Sciences, Patna, Bihar, India. All the patients included in this study were of mixed ages (ranging from 20 to 50 years), were from both sexes, were rK39 and direct agglutination test positive, and were human immunodeficiency virus (HIV) negative. The parasite burden in splenic aspirates was determined using the method described previously by Chulay and Bryceson (7). Brief profiles of the patients are presented in Tables 1 and 2. Monocytes from peripheral blood mononuclear cells from KA patients were isolated by adherence of the cells on a plastic petri dish and used for further experimentation.

Treatments. At 22 h postinfection, the Mφ (1×10^6) were treated with SAG (10 μg/ml in terms of Sb content) (33). In some experiments, cells were treated with verapamil (2.5 μM) (50) and/or probenecid (200 μM) (21) or lovastatin (10 μM) (52) for 1 h prior to treatment with SAG. Viability was tested for each inhibitor dose by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (data not shown) and found to be nontoxic under experimental conditions.

EAC/Dox cells and CEM-ADR5000 cells were treated with verapamil and/or probenecid or lovastatin at above-mentioned doses 1 h prior to treatment with

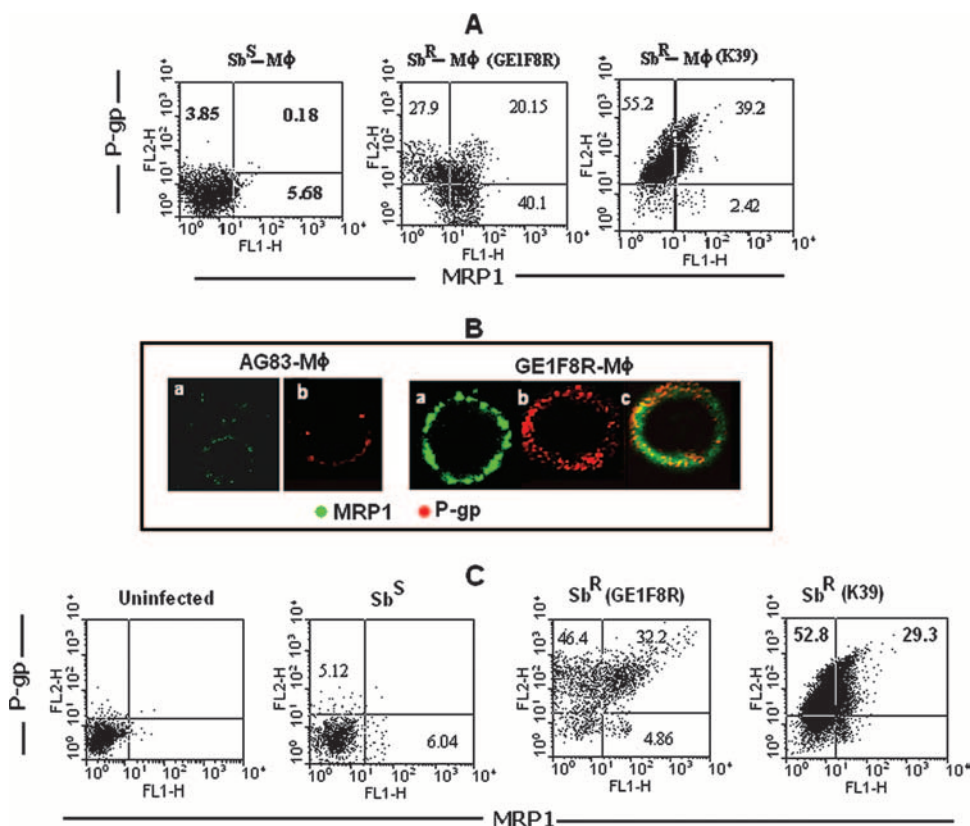


FIG. 2. Levels of expression of P-gp and MRP1 in murine peritoneal Mφ following in vitro infection and splenic Mφ following in vivo infection with Sb^S *L. donovani* (AG83) and Sb^R *L. donovani* (K39/GE1F8R). (A) Status of expression of P-gp and/or MRP1 in peritoneal Mφ of BALB/c were analyzed by flow cytometry following in vitro infection with Sb^S or Sb^R *L. donovani*. Representative data demonstrates enhanced expression of P-gp and/or MRP1 in Sb^R-Mφ compared to that in Sb^S-Mφ. The status of infection was checked in parallel sets by Giemsa staining, and it was found that more than 85% cells were infected, with infection loads being 1,152, 1,418, and 1,258 amastigotes per 100 Mφ for AG83, GE1F8R, and K39 infections, respectively, (data not shown). (B) Expression statuses of MRP1 (in green) and P-gp (in red) were studied in Sb^S-Mφ and Sb^R-Mφ (GE1F8R) by confocal microscopy. (C) Flow cytometric analysis of the status of MRP1 and P-gp expression in splenic Mφ derived from BALB/c mice following 2 months of infection with Sb^S *L. donovani* or Sb^R *L. donovani* showing the frequency of P-gp and MRP1 single- and double-positive cells. Splenic Mφ derived from uninfected BALB/c mice were kept as controls. Results are representative of five independent experiments.

either SAG (10 μg/ml) or Dox (15 μg/ml; equivalent to ~26 μM of Dox hydrochloride).

GE1F8R-infected hamsters (or K39-infected hamsters) were divided into six groups (I to VI). Each group consisted of 20 hamsters. Group I of both types (GE1F8R and K39) of Sb^R *L. donovani*-infected hamsters was the infected group that was untreated. Hamsters in groups II to VI received SAG injections (250 mg/kg of body weight per intramuscular shot) twice a week for 4 weeks (28). Hamsters from group II received SAG only. Group III received an oral treatment of probenecid (10 mg/kg) (47, 13), group IV received an oral treatment of verapamil (20 mg/kg) (9), group V received an oral treatment of a combination of 20 mg/kg of probenecid and verapamil and 10 mg/kg of probenecid, and group VI received an oral treatment of lovastatin (10 mg/kg) (11). Verapamil and/or probenecid or lovastatin was administered orally 3 h prior to each intramuscular administration of SAG. Ten animals from each group were sacrificed 30 days after the completion of treatment, and their hepatic and splenic parasite burdens were enumerated by the limiting dilution assay. The rest of the animals were monitored for a survival study, with adequate care as per the regulations of the institutional animal ethics committee. The experiment on survival was terminated after 240 days, and the surviving animals were sacrificed and checked for the presence of residual parasites in their bone marrow, spleens, and livers by limiting dilution (4).

Measurement of ROS. The level of ROS (includes superoxide, hydrogen peroxide, and other reactive oxygen intermediates) was monitored using the cell-permeable, nonpolar, H₂O₂-sensitive probe 2',7'-dichlorodihydrofluorescein diacetate as described before (33).

Measurement of NO. NO generation in response to SAG treatment was monitored by Griess reaction as described previously (33).

Quantification of intracellular antimony. Total intracellular Sb contents were measured in SAG (10 μg/ml)-treated cells by electrothermal atomic absorption spectroscopy as described previously (43) with minor modifications.

Detection of expression of MRP1 and P-gp. The levels of expression of MRP1 and P-gp in Mφ or peripheral blood monocytes of the patients were determined by immunostaining, followed by flow cytometry (Becton Dickinson) and/or confocal microscopy (LSM 510; Carl Zeiss, Jena, Germany), as described earlier (6). The appropriate isotype control was used for each individual case. Since these antibodies were found to recognize MRP1-like molecules in GE1F8R and P-gp-like molecules in K39 promastigotes, all stainings were performed 18 to 24 h after washing in cold the unpermeabilized infected cells to specifically check the expression of P-gp and MRP1 on the host cell surface. Hence, no counterstaining with DNA binding dyes like DAPI (4',6'-diamidino-2-phenylindole) or propidium iodide was performed even after fixation of the cells. No epitopes were recognized in AG83 promastigotes.

Dox retention assay. The Dox retention assay was performed using EAC/Dox cells following the method described previously (52) with slight modifications. In brief, cells were treated with different concentrations of lovastatin for 1 h at 37°C in 5% CO₂ in air. After that, Dox (15 μg/ml; equivalent to ~26 μM of Dox hydrochloride) was added and incubated further for 3 h. Cells were harvested in phosphate-buffered saline and washed three times in phosphate-buffered saline to remove excess Dox. After 30 min, the amount of Dox retained was analyzed in a flow cytometer (Becton Dickinson) and observed under a laser scanning microscope (LSM 510; Carl Zeiss, Jena, Germany).

Statistical analysis. Each experiment was performed three to five times, and results are expressed as means ± standard errors. The Student *t* test for significance was performed, and a *P* value of <0.05 was considered significant. Anal-

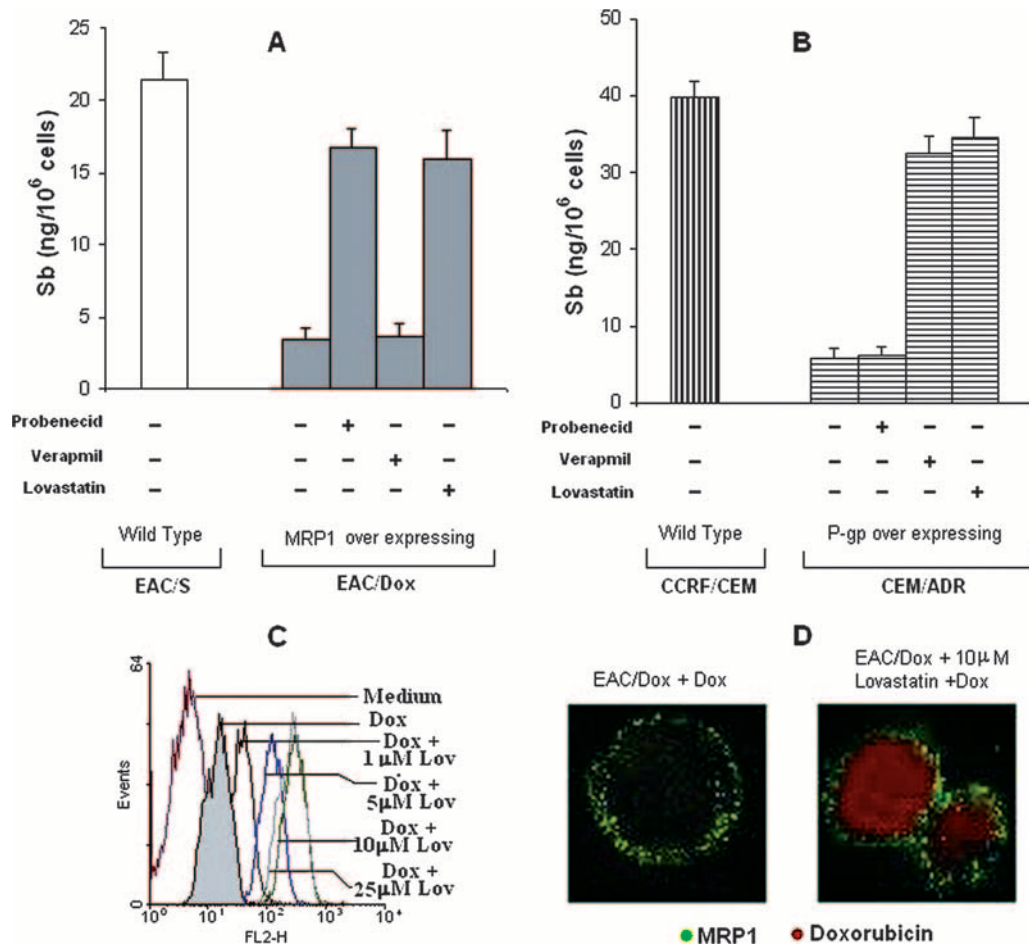


FIG. 3. Effects of verapamil and/or probenecid or of lovastatin on the drug retention ability of MRP1-overexpressing murine breast carcinoma cell line EAC/Dox and P-gp-overexpressing human lymphoblastic leukemia cell line CEM-ADR 5000. Ability of Sb retention in (A) MRP1-overexpressing EAC/Dox and (B) P-gp-overexpressing CEM-ADR5000 cell lines and their parental counterparts, i.e., EAC/S (lacking overexpression of MRP1) and CCRF/CEM (lacking overexpression of P-gp), respectively, was estimated at 6 h post-SAG treatment (10 μ g/ml) in the presence or the absence of 200 μ M of probenecid (inhibitor of MRP1) or 2 μ M of verapamil (inhibitor of P-gp) or 10 μ M of lovastatin. (C) Inhibitory effect of lovastatin (lov) on MRP1-mediated transport was assessed by the Dox retention assay. An increase in retention of Dox by EAC/Dox cells with increasing dose of lovastatin treatment (1 h prior to Dox treatment) was evident from flow cytometry. (D) Retention of Dox (as shown by red fluorescence) by MRP1 overexpressing (as shown by green fluorescence) EAC/Dox cells in the presence of an optimum dose of lovastatin (10 μ M) was observed by confocal microscopy. Results are presented as means \pm standard errors of the means of five independent experiments (A and B) or as representative of four independent experiments (C and D). Error bars indicate standard deviations. -, absence of; +, presence of.

ysis of variance with a posttest correction for multiple groups was used for statistical analysis of the data, and a *P* value of <0.05 was considered significant. Representative data of flow cytometry and fluorescence microscopy of at least three independent experiments are presented.

RESULTS

Clearance of Sb from M ϕ . Previously we have shown that SAG induces the generation of leishmanicidal molecules like ROS through activation of the PI3K-PKC-extracellular signal-regulated kinase 1/2 pathway in early phase (6 h posttreatment) and NO through activation of the PI3K-Akt-p38 mitogen-activated protein kinase pathway in late phase (24 h posttreatment) in Sb^s *L. donovani*-infected M ϕ (Sb^s-M ϕ), leading to the elimination of intracellular *L. donovani* parasites (33). However, SAG not only failed to eliminate intracellular Sb^r *L. donovani* (Fig. 1A) in M ϕ infected with Sb^r *L. donovani* (Sb^r-M ϕ) but also failed to induce the generation of ROS (Fig.

1B) and NO (Fig. 1C). Since the efflux of drug from the cell is well reported to be an important mechanism of drug resistance, especially in cancer (23), we measured the total intracellular Sb content in M ϕ infected with SAG-sensitive and -resistant *L. donovani* strains. The Sb content in Sb^s-M ϕ increased sharply following SAG treatment, reaching a steady-state level at ~6 h posttreatment. On the contrary, Sb content in M ϕ infected with Sb^r *L. donovani* strains, i.e., GE1F8R (GE1F8R-M ϕ) and K39 (K39-M ϕ), increased slightly until 20 min post-SAG treatment and rapidly declined thereafter, reaching the basal level (Fig. 1D). This suggested that in contrast to Sb^s-M ϕ , Sb^r-M ϕ were unable to retain Sb intracellularly upon SAG treatment.

Expression of MRP1 and P-gp in M ϕ following infection. Overexpression of ABC transporters like P-gp or MRP1 causes multiple drug resistance in different diseases (5) like cancers (23, 30), HIV infection, or mycobacterium-HIV coinfection (22, 3).

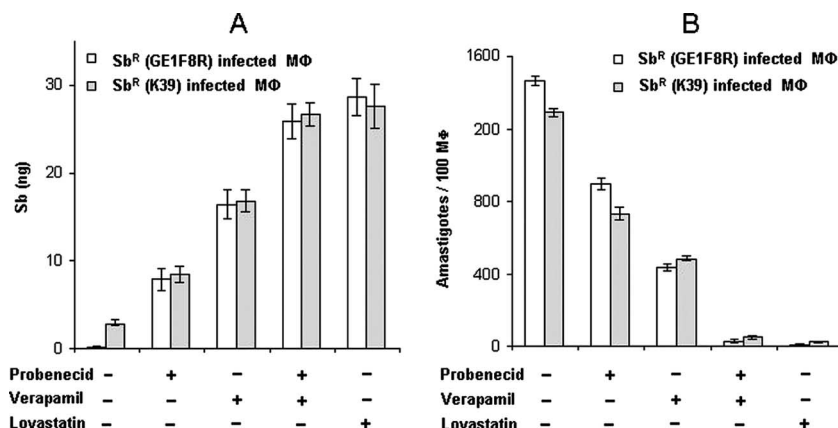


FIG. 4. Effects of verapamil and/or probenecid or of lovastatin on the Sb retention ability of Sb^f-Mφ and SAG-mediated killing of intracellular parasite. (A) Effect of verapamil (2 μM) and/or probenecid (200 μM) or lovastatin (10 μM) on the ability of intracellular Sb retention in Sb^f-Mφ. (B) Intracellular parasite number was determined for Sb^f-Mφ in the presence and the absence of verapamil (2 μM) and/or probenecid (200 μM) or of lovastatin (10 μM) at 24 h following SAG treatment. An inverse relationship was obtained between Sb retention and killing of intracellular parasites upon pretreatment with a combination of verapamil and probenecid or with lovastatin. Error bars indicate standard deviations. -, absence of; +, presence of.

Therefore, we analyzed the expression status of MRP1 and P-gp in host cells, i.e., in Sb^f-Mφ and Sb^s-Mφ. We observed an increased number of MRP1-positive and P-gp-positive cells in the cases of both GE1F8R-Mφ and K39-Mφ, with frequent coexpression of both transporters compared to the case for Sb^s-Mφ (Fig. 2A). This flow cytometric observation was further confirmed by confocal microscopy of GE1F8R-Mφ, in which various degrees of P-gp and MRP1 colocalization were observed (Fig. 2B).

In order to confirm these observations in an *in vivo* experimental setting, the expression levels of MRP1 and P-gp were tested in splenic Mφ from BALB/c mice infected with either Sb^s *L. donovani* or Sb^f *L. donovani*. The splenic Mφ population derived from Sb^f *L. donovani*-infected mice showed a remarkable increase in P-gp single-positive (Fig. 2C) and MRP1-P-gp double-positive cells (Fig. 2C) compared to the number of cells from Sb^s *L. donovani*-infected mice.

Involvement of ABC transporters in Sb clearance. To determine whether higher expression levels of P-gp and MRP1 are indeed involved in the nonretention of Sb, four cell lines, viz., MRP1 overexpressing EAC/Dox (31, 32), P-gp overexpressing CEM-ADR5000 (14), and their respective parental Dox-sensitive counterparts EAC/S and CCRF/CEM, were treated with SAG for 6 h and total intracellular Sb contents were measured. Unlike their parental counterparts, both EAC/Dox and CEM/ADR5000 failed to retain Sb (Fig. 3A and B). Furthermore, EAC/Dox retained Sb following SAG treatment in the presence of the MRP1 inhibitor probenecid (18, 21) but not in the presence of verapamil (Fig. 3A), while CEM/ADR5000 retained Sb in the presence of the P-gp-inhibitory isoform of verapamil (26, 49, 50) but not in the presence of probenecid (Fig. 3B). Thus, drug sensitivity correlates with ABC transporter expression and strongly suggests a selective involvement of P-gp and MRP1 in Sb clearance from EAC/Dox and CEM/ADR5000 lines, respectively. Interestingly, both EAC/Dox and CEM/ADR5000 cell lines retained Sb when treated with SAG in the presence of lovastatin (Fig. 3A and B). These results indicate that in addition to its previously reported P-gp-inhibitory effect (52), lovastatin may also inhibit

MRP1. To confirm this possibility, we performed a Dox retention assay in EAC/Dox in the presence of lovastatin. Pretreatment of EAC/Dox with lovastatin for 60 min before being loaded with Dox resulted in a dose-dependent increase in the retention of Dox, which reached a plateau at a 10-μM lovastatin dose (Fig. 3C). Confocal microscopy further confirmed that Dox is retained in EAC/Dox cells in the presence of lovastatin without an observable change in MRP1 expression (Fig. 3D). Thus, lovastatin exerts an inhibitory effect on both MRP1-mediated and P-gp-mediated transport. Therefore lovastatin stands out as an excellent candidate as RMA that could induce not only the retention of Sb but also that of an unrelated chemotherapeutic agent such as Dox in cancer cells.

Effect of inhibition of MRP1 and P-gp in Sb^f-Mφ. As pretreatment with inhibitors of ABC transporters resulted in the accumulation of Sb, these RMAs were tested for their abilities to restore Sb retention in Sb^f-Mφ upon SAG treatment. Pretreatment of Sb^f-Mφ with a combination of verapamil and probenecid or with lovastatin significantly allowed Sb retention ($P < 0.001$) even by 24 h after SAG treatment (Fig. 4A). The treatment induced ROS generation at 6 h and NO at 24 h (data not shown) and resulted in SAG-mediated killing of intracellular Sb^f *L. donovani* (Fig. 4B). The combination of verapamil and probenecid was more effective than single inhibitors alone were, allowing clearance of more than 90% of the parasite by 24 h of SAG treatment. Pretreatment with lovastatin alone resulted in a more than 95% elimination of intracellular Sb^f *L. donovani* following SAG treatment (Fig. 4B). This result confirms that the inhibitions of both P-gp and MRP1 are necessary to prevent Sb efflux and promote SAG-mediated parasite clearance.

Inhibitors of ABC transporters as RMAs. Since experimental VL in hamsters closely mimics KA in humans, inhibitors of P-gp and MRP1 as potential RMAs were evaluated in hamsters infected with Sb^f *L. donovani*. GE1F8R-infected or K39-infected hamsters were divided into six groups (I to VI) and received treatments as described in Materials and Methods. GE1F8R-infected hamsters showed hardly any reduction of

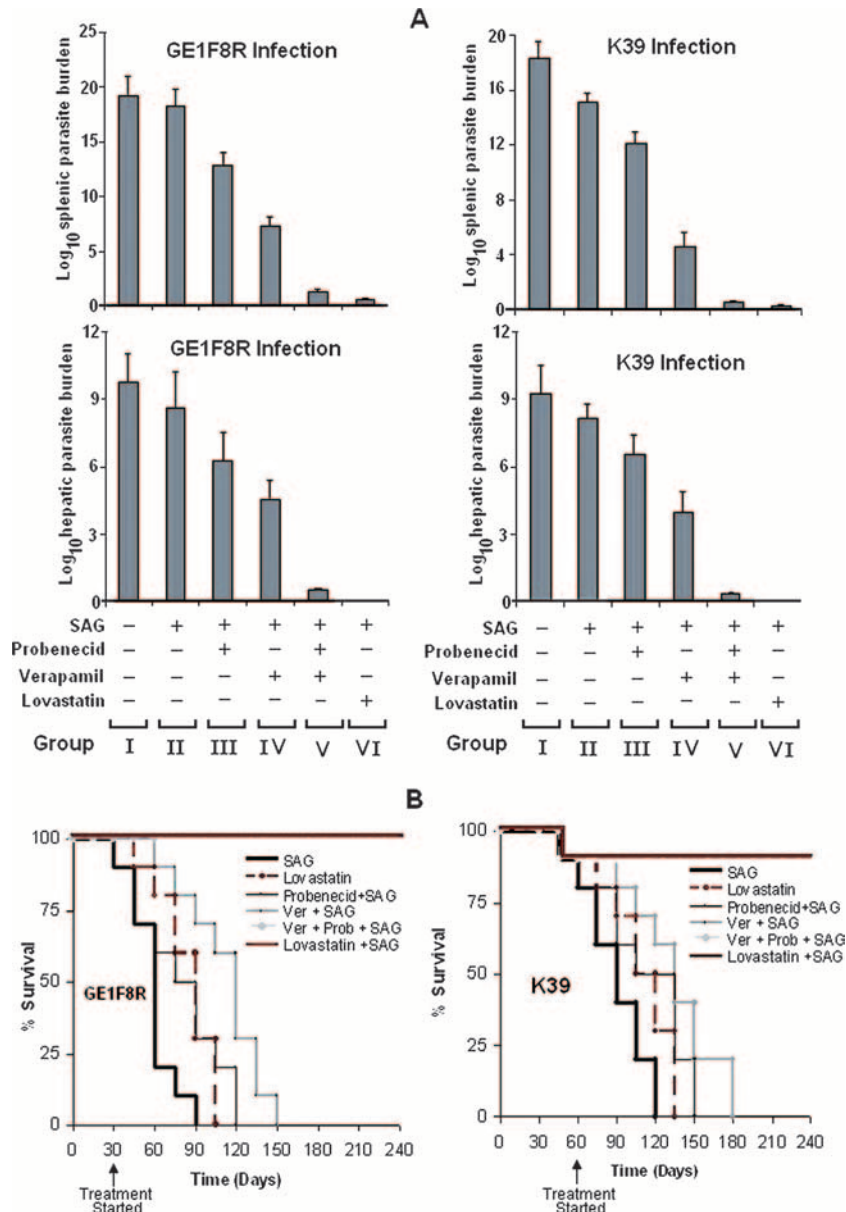


FIG. 5. Oral administration of probenecid and/or verapamil or of lovastatin prior to SAG treatment in *Sb*¹ *L. donovani*-infected hamsters offered protection. GE1F8R (30 day)- and K39 (60 day)-infected hamsters were used because of their essentially equal parasite burdens in spleens at the two time points. Both GE1F8R-infected and K39-infected hamsters were divided into six groups (I to VI). All the groups (20 in each group) except group I (the untreated infected group) received SAG injections (two injections [250 mg/kg per shot] per week for 4 weeks). Groups III to VI also received other treatments, together with SAG. Groups III, IV, V, and VI received oral treatments of probenecid, verapamil, verapamil plus probenecid, and lovastatin, respectively, 3 h prior to each SAG injection. Ten animals from each group were sacrificed 30 days after the last SAG treatment. Parasite burdens of spleens and livers were enumerated by limiting dilution and were expressed as log₁₀ parasite burden (A). In each of these experiments, additional groups of animals were taken to test the efficacies of treatments with inhibitors alone. In these groups, the splenic parasite burdens were $\sim 2 \times 10^{15}$ (treated with only probenecid), $\sim 8 \times 10^{14}$ (treated with only verapamil), $\sim 7 \times 10^{14}$ (treated with only probenecid plus verapamil), and $\sim 2 \times 10^{14}$ (treated with only lovastatin). Ten animals from each group (I to VI) were kept for a study of survival kinetics up to 240 days (B). Groups I (untreated infected) and II (treated with only SAG) showed similar survival kinetics; therefore, the result for group I is not presented. Asterisks indicate the termination of experiment. Error bars indicate standard deviations. -, absence of; +, presence of.

parasite burden upon SAG treatment (Fig. 5A), whereas similar treatment of K39-infected hamsters caused an approximately 15% reduction in parasite burdens in spleens, confirming that these two *L. donovani* parasite strains are indeed SAG resistant in an in vivo setting. Treatment with probenecid

(group III) led to SAG-mediated reductions in spleen and liver parasite burdens by $\sim 27\%$ and $\sim 35\%$, respectively, in GE1F8R-infected hamsters and by 33% and 23%, respectively, in K39-infected hamsters compared to values for the untreated infected group (group I). Interestingly, group IV hamsters,

TABLE 3. Organ sterile protection in hamsters receiving verapamil plus probenecid or lovastatin prior to SAG injection

Group	<i>L. donovani</i> strain used for infection	No. of hamsters with sterile protection of organ ^a	
		Spleen	Liver
V	GE1F8R	8	9
	K39	9	9
VI	GE1F8R	9	10
	K39	10	10

^a Ten hamsters were used to determine the level of organ sterile protection. Protection was determined by the limiting dilution method.

which received verapamil plus SAG, showed significantly more reduced parasite burdens in both spleens and livers compared to the burdens for hamsters treated with SAG only ($P < 0.001$). Oral treatment with a combination of probenecid and verapamil (group V) or with lovastatin (group VI) prior to SAG treatment showed reductions of more than 90% (about 10^{17} -fold) and more than 95% (about 10^{18} -fold), respectively, in both hepatic and splenic parasite burdens (Fig. 5A) and offered sterile protection in spleens and livers in the majority of GE1F8R-infected and K39-infected hamsters (Table 3).

The survival rate was studied for each experimental group until day 240 was reached (Fig. 5B). Data from group I was similar to that from group II and is not shown. All GE1F8R-infected and K39-infected hamsters belonging to groups I and II died by 90 days and 120 days, respectively, after infection. Survival of both GE1F8R-infected and K39-infected hamsters could be improved only to some extent with either probenecid or verapamil treatment prior to each SAG treatment, while all animals belonging to groups V (probenecid plus verapamil plus SAG) and VI (lovastatin plus SAG) survived until the termination of the experiment (at 240 days) (Fig. 5B). After sacrifice, no residual parasites could be detected in livers, spleens, and bone marrow by the limiting dilution assay. Thus, oral treatment with a combination of verapamil and probenecid or with lovastatin allowed SAG-mediated cure of VL in animals infected with Sb^r *L. donovani*.

Animals treated with only inhibitors did not show major changes in the course of infection, although the survival of the group treated with only verapamil plus probenecid increased slightly (approximately 70% of the animals treated with only verapamil plus probenecid died within 90 days postinfection in the case of GE1F8R infection and within 120 days postinfection in the case of K39 infection [data not shown]). Interestingly, about 60% of the animals treated with only lovastatin survived until 75 days postinfection in the case of GE1F8R infection and 105 days postinfection in the case of K39 infection (Fig. 5), indicating that only lovastatin treatment slows down the progression of the disease.

Expression of ABC transporters in monocytes of KA patients. In order to evaluate the role of ABC transporters in KA patients, we analyzed the status of P-gp and MRP1 expression and Sb accumulation following in vitro SAG treatment of peripheral blood monocytes from 10 confirmed SAG-responsive KA (Sb^s-KA) and 10 confirmed SAG-unresponsive KA (Sb^r-KA) patients. Monocytes of all Sb^r-KA patients not only had

much higher glutathione contents (data not shown) but also expressed significantly higher levels of P-gp and MRP1 compared to those of Sb^s-KA patients ($P < 0.01$), although the levels of expression of MRP1 and P-gp varied from patient to patient (Fig. 6). When the monocytes from these patients were treated with SAG in vitro, the cells from confirmed Sb^s-KA patients showed significantly higher Sb retention levels (149 ± 9 ng/ 10^6 cells) than those of cells from confirmed Sb^r-KA patients (22.8 ± 10 ng/ 10^6 cells) ($P < 0.005$). Pretreatment with verapamil plus probenecid restored Sb retention by Sb^r-KA monocytes upon SAG treatment (Fig. 6). We further extended our studies with peripheral blood monocytes of fresh KA patients from areas of India where SAG responsiveness (SAG-responsive area) and unresponsiveness (SAG-unresponsive area) are found. Interestingly, though the levels of expression of MRP1 and P-gp and the frequencies of coexpressing cells varied, monocytes of fresh KA patients from SAG-unresponsive areas showed significantly higher ($P < 0.01$) levels of expression of P-gp and MRP1 compared to those of fresh KA patients from SAG-responsive zones. Again, monocytes of fresh KA patients from SAG-responsive zones showed significantly higher Sb retention levels (173 ± 10 ng/ 10^6 cells) ($P < 0.005$) compared to those of patients from SAG-unresponsive areas (30.8 ± 8 ng/ 10^6 cells) (Fig. 6).

To examine the Sb sensitivity, parasites were isolated from splenic aspirates of fresh (i.e., untreated) KA patients, propagated in culture, and used to infect M ϕ from normal BALB/c mice. More than 90% of intracellular parasites derived from KA patients from Sb-sensitive areas were eliminated by 10 μ g/ml SAG in vitro, whereas a similar dose of SAG eliminated less than 50% of the intracellular parasites isolated from KA patients from Sb-resistant areas (Fig. 7A). Moreover, in contrast to the case for parasites derived from a KA patient from a Sb-sensitive area, infection with parasites from Sb-unresponsive areas could upregulate P-gp and MRP1 of M ϕ derived from BALB/c mice (Fig. 7B).

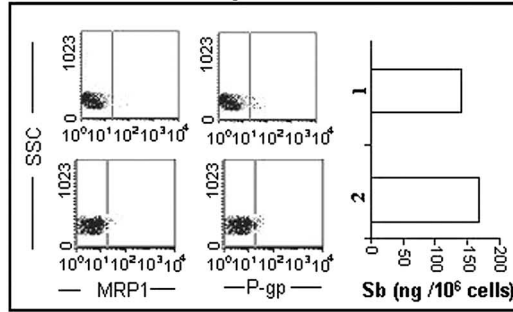
Role of parasite antigen in upregulation of expression of host ABC transporters. Since peripheral blood monocytes of the Sb^r-KA patients do not harbor demonstrable parasites at the active stage of the disease but still possess upregulated P-gp and MRP1 expression, it is likely that soluble and circulating parasite antigens cause an upregulation of the expression of these transporters. To test this hypothesis, we conducted an overnight incubation of uninfected M ϕ from normal BALB/c mice with crude antigens from sonicated Sb^r *L. donovani* strains (GE1F8R or K39). We observed that Sb^r *L. donovani* parasite antigens could induce the upregulation of MRP1 and P-gp in uninfected M ϕ (Fig. 8A). Moreover, the incubation of uninfected M ϕ with Sb^r *L. donovani* antigens (but not with Sb^s *L. donovani* antigens) resulted in decreased intracellular Sb (total) accumulation following SAG treatment (Fig. 8B).

DISCUSSION

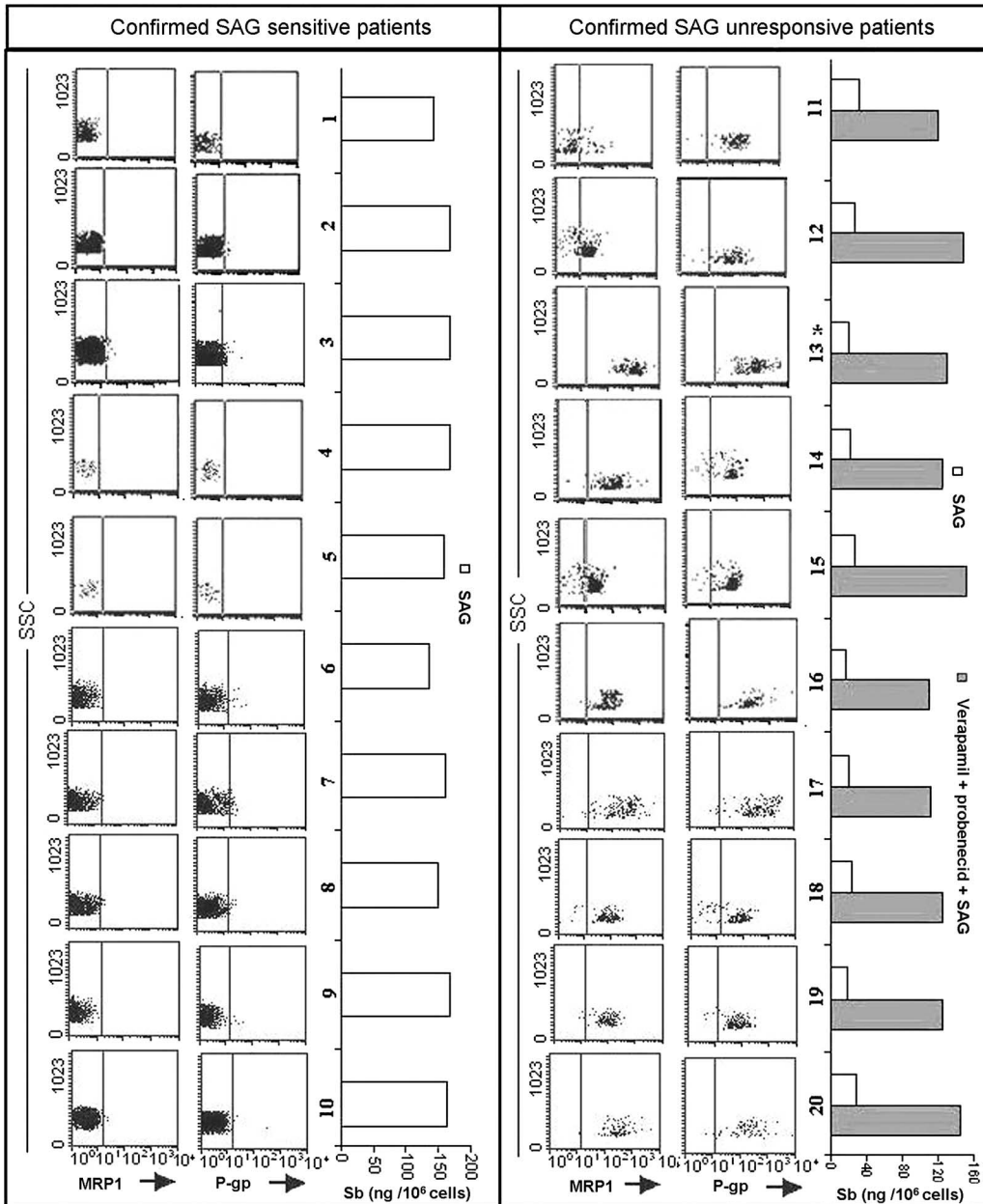
Drug resistance is the major impediment to successful chemotherapy in several diseases like cancer and AIDS and in different parasitic diseases, including leishmaniasis. In the case of leishmaniasis, resistance to antimonials has emerged as the major pitfall in treatment in view of limited alternative treat-

A

Healthy volunteers



B



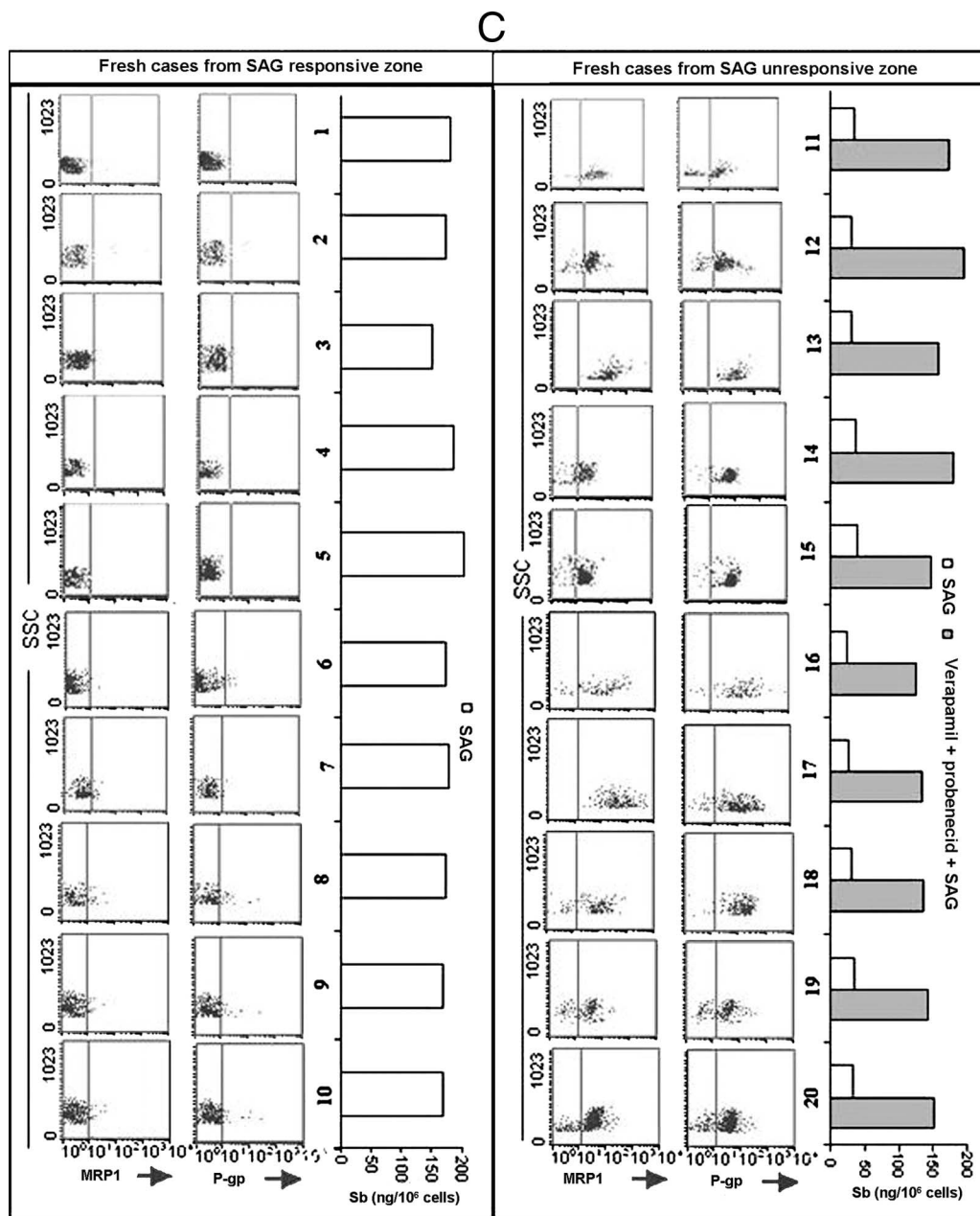


FIG. 6. Status of MRP1 and P-gp expression and the Sb retention ability of monocytes of confirmed SAG-sensitive and SAG-unresponsive KA patients as well as of fresh KA patients from SAG-unresponsive and -sensitive zones. Expression status of MRP1 and P-gp and Sb retention in monocytes of (A) healthy volunteers, (B) 10 confirmed SAG-sensitive KA patients (Sb^s-KA), and 10 unresponsive KA patients (Sb^r-KA) were studied by flow cytometry. Intracellular Sb retention was analyzed by atomic absorption spectroscopy 6 h post-SAG treatment. Monocytes of Sb^s-KA patients showed much lower frequencies of MRP1-positive and P-gp-positive cells and retained much higher amounts of Sb 6 h following SAG treatment compared to results for Sb^r-KA monocytes. Treatment of all Sb^r-KA monocytes with a combination of verapamil (2 μM) and probenecid (200 μM) 1 h prior to SAG treatment allowed substantial Sb retention. Details of the patients are described in Table 1. (C) Expression status of MRP1 and P-gp and Sb retention ability of monocytes of 20 fresh KA patients, of which 10 came from the SAG-sensitive zone and the rest from the SAG-resistant zone. Identical studies were performed as with Sb^r-KA and Sb^s-KA monocytes. Details of the patients are described in Table 2. For SSC, 1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate.

ments, reports of multiple drug resistance (8, 41, 42, 48), and lack of effective vaccination. Previous studies emphasizing the expression of analogs of ABC transporters by *Leishmania* promastigotes, however, do not fully explain the resistance displayed by amastigote forms within human hosts, especially when SAG also activates the host's immune system that helps

to eliminate intracellular parasite. We show here that resistant parasites strongly increase expression of the host's P-gp and MRP1 transporters on the surfaces of infected Mφ, resulting in Sb clearance from the host cells in the course of in vitro as well as in vivo experimental infection. Moreover, studies performed on patient samples from Sb-resistant infection areas unequi-

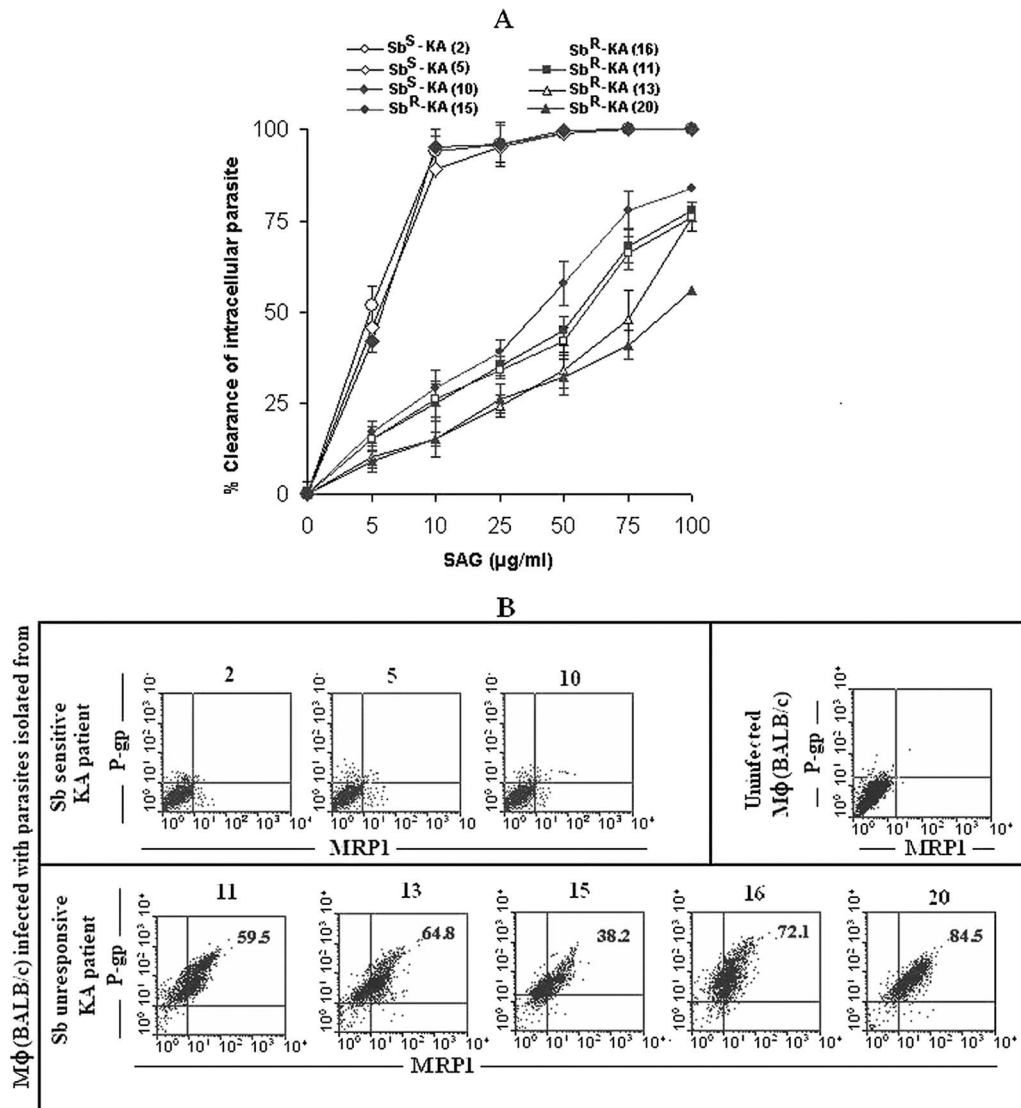


FIG. 7. Status of SAG sensitivity and ability of induction of MRP1 and P-gp expression of parasites isolated from splenic aspirates of KA patients from SAG-sensitive and SAG-unresponsive zones. (A) Normal BALB/c Mφ were infected with promastigotes (the ratio of Mφ versus promastigote was 1:10) cultured from splenic aspirates of KA patients from SAG-sensitive (Sb^S) or -unresponsive (Sb^R) areas (patient number as per Table 2 is given). After 22 h, more than 85% cells were infected ($1,288 \pm 167$ Sb^R parasites/100 Mφ and $1,056 \pm 124$ Sb^S parasites/100 Mφ). Following infection in Mφ, parasites isolated from patients belonging to Sb^R areas were much less sensitive to SAG (50% inhibitory concentration, 63.6 ± 17.9 $\mu\text{g/ml}$) compared to parasites isolated from patients belonging to Sb^S areas (50% inhibitory concentration, 5.5 ± 0.5 $\mu\text{g/ml}$). (B) Parasites of patients from Sb^R areas could induce high levels of expression of MRP1 and/or P-gp in host Mφ in contrast to those induced by parasites of patients from Sb^S areas. Results are presented as means \pm standard errors (error bars) of the means of five independent experiments (A) or are representative of five independent experiments (B).

vocally indicate that a similar phenomenon occurs during natural human infection. Of course, this does not exclude the possibilities that analogs of mammalian ABC transporters on amastigotes also play some role in drug resistance and inhibitors of mammalian ABC transporters may also inhibit these molecules of the parasite to allow better action of SAG. However, upregulation of ABC transporters in host cells may act as the foremost barrier for drugs and, thus, the transporters are of considerable interest.

As parasites replicate in Mφ, we focused our study on these cells. However, this focus does not exclude an upregulation of these transporters in other cell types like lymphocytes. The

ability of a leishmanial antigen to upregulate expression of MRP1 has been reported earlier for Th1 clones (29). Indeed, it is well established that monocytes do not harbor parasites at the active stage of the disease. In spite of this finding, peripheral blood monocytes from Sb^R -KA patients upregulate P-gp and MRP1. Therefore, it is likely that soluble and circulating parasite antigens can cause an upregulation of expression in these transporters. This result is supported by our findings that formalin-fixed Sb^R *L. donovani* or even extracts from Sb^R *L. donovani* strains can induce the upregulation of MRP1 and P-gp in uninfected murine Mφ and reduce Sb accumulation following SAG treatment. Thus, the resistance mechanism may

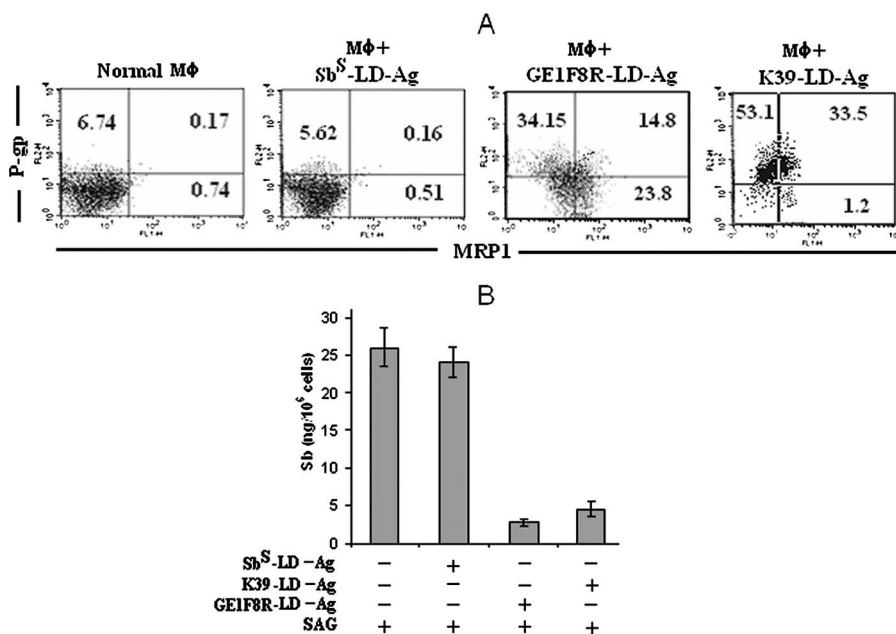


FIG. 8. Effect of incubation of normal murine Mφ with *L. donovani* parasite antigen. Normal BALB/c Mφ (10⁶) were incubated overnight with crude antigens derived by sonication of 10⁷ Sb^S or Sb^F *L. donovani* (K39 or GE1F8R), excess antigen was washed with RPMI, (A) the status of MRP1 and P-gp was checked, (B) mice were treated with SAG (10 μg/ml), and Sb content was measured at 24 h post-SAG treatment. Antigen (Ag) prepared from Sb^S *L. donovani* (AG83) is presented as Sb^S *L. donovani*-Ag and those from GE1F8R (Sb^F *L. donovani*) and K39 (Sb^F *L. donovani*) parasites are presented as GE1F8R-*L. donovani*-Ag and K39-*L. donovani*-Ag, respectively. Results are presented as representative of five independent experiments (A) or as means ± standard errors (error bars) of the means of five independent experiments (B). -, absence of; +, presence of.

operate in different cells of parasite reservoirs, even in the absence of parasite replication in situ. The identification of *L. donovani* parasite antigens through proteome mapping (20) and an analysis of their glycosylinositol phospholipids responsible for this upregulation are currently under investigation. It also will be important to determine whether such antigens are displayed on the surfaces of infected Mφ and sensed by adjacent Mφ or whether they are released from infected Mφ phagolysosomes to trigger the overexpression of ABC transporters. However, the importance of Sb^F *L. donovani* antigens in SAG resistance through an upregulation of expression in ABC transporters is further demonstrated by our observation that SAG fails to eliminate intracellular Sb^S *L. donovani* from Mφ that have been preincubated with Sb^F *L. donovani* antigens and then infected in vitro with Sb^S *L. donovani* (data not shown).

Our results show that inhibitors of P-gp and MRP1 could restore sensitivity toward Sb not only in vitro but also in vivo. Sb^F *L. donovani* infection in golden hamsters is fatal, even upon SAG treatment. The survival of animals infected with Sb^F *L. donovani* and the clearance of SAG-resistant parasites when treated with SAG plus either a combination of verapamil plus probenecid or lovastatin suggest major roles for P-gp and MRP1 in drug efflux. Thus, our results clearly show that inhibitors of P-gp and MRP1 represent important new therapeutic tools to combat Sb resistance. Lovastatin is a well-tolerated drug (12), and it will be important to evaluate its beneficial effect as an RMA in clinical trials dealing with SAG treatment of VL patients. Finally, lovastatin alone could inhibit both P-gp-mediated and MRP1-mediated transport to allow the re-

tention of antimonials as well as Dox in drug-resistant cancer cells, thereby opening up new opportunities for the treatment of other infectious diseases and cancers where drug resistance is a major challenge and often involves the overexpression of P-gp and MRP1.

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