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In Vitro Susceptibility of Field Isolates of *Leishmania donovani* to Miltefosine and Amphotericin B: Correlation with Sodium Antimony Gluconate Susceptibility and Implications for Treatment in Areas of Endemicity▽

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Indian *Leishmania donovani* isolates ($n = 19$) from regional zones representing various levels of antimony resistance displayed significantly ($P < 0.01$) correlated results with respect to in vitro susceptibility to the antileishmanial drugs sodium antimony gluconate, amphotericin B, and Miltefosine, raising the possibility of cross-resistance mechanisms operating in the field isolates. The results of gene expression analysis of LdMT and LdRos3 were suggestive of alternate mechanisms of Miltefosine susceptibility in the isolates.

A high (>60%) proportion of non-antimony-responsive cases of Kala azar in India and the anthroponotic mode of transmission of the parasite causing the disease increase the chances of the generation and spreading of drug-resistant parasites (15, 17). The second-line antileishmanials amphotericin B (AmB) and Miltefosine (MIL) are highly effective for treatment of antimony-resistant patients but are of limited utility because of adverse reactions and high cost. A recent report of unresponsiveness to Ambisome in Sudanese patients of VL is worrisome and indicates the emergence of AmB-resistant parasites (9). Preliminary data from a phase IV trial with MIL suggested a doubling of the relapse rate, indicating lower drug efficacy than in phase II and III trials and providing a warning about the emergence of resistance (3, 18, 19).

Earlier studies using isolates from responsive and nonresponsive patients indicated that resistance to antimonials is an intrinsic property of the parasite (4, 8, 15, 16). Antimony resistance varies among zones representing differing levels of endemicity, emphasizing the acquired nature of resistance in the region (15). Sodium antimony gluconate (SAG)-resistant isolates exhibited cross-resistance to AmB and MIL, with HSP83 and a calpain-related protein being implicated in resistance by modulating drug-induced programmed cell death (21). Since the use of MIL for VL treatment has been introduced only recently, resistance has not yet been reported in the field; however, a wide range of 50% effective doses (ED_{50}) of MIL has been observed for parasite isolates from Nepal and Peru (23). The results of earlier studies revealed a role in MIL uptake and susceptibility for the LdMT-LdRos3-dependent flippase machinery at the plasma membrane (10–12). The present study was aimed at (i) evaluating the in vitro natural susceptibility of field isolates of *Leishmania donovani* to SAG, AmB, and MIL and (ii) correlating MIL susceptibility with the mRNA expression of LdMT and LdRos3 to explore their role in MIL resistance and potential as markers of MIL resistance in field isolates.

The present study considered 19 *L. donovani* isolates from VL patients representing regional zones with various degrees of disease endemicity. In vitro susceptibility of parasites from SAG-treated patients (responsive and nonresponsive) and AmB-treated patients (all responded to treatment, and no clinical resistance was observed) was studied. Informed consent based on the guidelines of the Ethical Committee, Safdarjung Hospital, New Delhi, India, was obtained from the patients. The field isolates were investigated for susceptibility to SAG (Albert David Ltd., India), AmB (Sigma), and MIL (Cayman Chemical Company) at the intracellular amastigote and promastigote stages as described previously (15). The clinical profiles of VL patients and in vitro susceptibilities of parasite isolates are summarized in Table 1.

For SAG, ED_{50} values ranged from 2.14 ± 0.28 (mean ± standard deviation) to 20.30 ± 0.84 μg/ml, with a mean ED_{50} of 12.18 ± 5.68 μg/ml. The mean ED_{50} of isolates from the high-resistance (HR) region (15.81 ± 2.50 μg/ml) was significantly ($P < 0.001$) higher than that of the low-resistance (LR)-region isolates (5.46 ± 3.69 μg/ml). We observed a strong correlation of in vitro SAG susceptibility with the endemicity zones ($r_{rank} = 0.998$) and with the clinical response ($r_{rank} = 0.982$), based on the criteria defined earlier (15, 20). The ED_{50} values for AmB at the amastigote stage ranged from 0.17 ± 0.01 (mean ± standard deviation) to 0.77 ± 0.08 μg/ml, with a mean of 0.39 ± 0.19 μg/ml. The ED_{90} values ranged from 0.37 ± 0.02 to 2.55 ± 0.42 μg/ml (mean, 1.29 ± 0.63 μg/ml). The mean ED_{50} (0.49 ± 0.17 μg/ml) of isolates from the HR region was significantly higher ($P < 0.001$) than the mean ED_{50} (0.21 ± 0.03 μg/ml) for LR zone isolates. At the promastigote stage, the ED_{50} for AmB ($n = 18$) ranged from 0.312 ± 0.014 to 1.62 ± 0.134 μg/ml (mean, 0.89 ± 0.44 μg/ml). Antileishmanial activity of AmB was partially correlated ($r = 0.596$) at the amastigote and promastigote stages.

The field isolates ($n = 19$) showed various levels of suscep-

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TABLE 1. Clinical profiles of VL patients from LR and HR regions and in vitro susceptibility of parasite isolates to SAG, AmB, and MIL, with expression indices of MIL transporters LdMT and LdRos3

Strain	Sex/age (yr)[a]	Area in India/region category or source and/or description	Treatment (response)[b]	Susceptibility (ED_{50} [μg/ml])[c]					Fold decrease in expression[d]	
				SAG (Amas)	AmB		MIL		LdMT	LdRoS3
					Amas	Pro	Amas	Pro		
*Ld*AG83		Standard Indian *L. donovani* strain		2.06 ± 0.23	0.017 ± 0.01	0.023 ± 0.01	0.85 ± 0.03	1.05 ± 0.07	1	1
K59	F/21	Vaishali/HR	SAG (NR)	14.66 ± 3.29	0.180 ± 0.02	0.50 ± 0.006	1.86 ± 0.07	1.19 ± 0.07	2.84 ± 0.22	4.06 ± 0.21
K131	M/22	Saharsha/HR	SAG (NR)	19.38 ± 1.68	0.35 ± 0.08	0.43 ± 0.035	0.51 ± 0.06	0.63 ± 0.03	2.43 ± 0.25	11.1 ± 0.23
K149	M/20	Saran/HR	AmB (R)	15.70 ± 4.01	0.28 ± 0.10	1.46 ± 0.12	1.57 ± 0.16	1.56 ± 0.11	1.08 ± 0.56	7.69 ± 0.16
K192	M/24	Saran/HR	AmB (R)	20.30 ± 0.84	0.67 ± 0.10	0.78 ± 0.12	1.99 ± 0.17	1.91 ± 0.08	0.23[e] ± 0.04	1.9 ± 0.13
K251	M/11	Saran/HR	ND[f]	11.82 ± 1.28	0.55 ± 0.01	1.42 ± 0.09	1.59 ± 0.07	0.40 ± 0.05	7.68 ± 0.10	52.63 ± 0.06
K417	F/8	Muzaffarpur/HR	AmB (R)	14.65 ± 0.67	0.44 ± 0.01	0.69 ± 0.007	1.90 ± 0.04	1.72 ± 0.11	1.88 ± 0.23	5.02 ± 0.23
K429	M/26	Saharsha/HR	AmB (R)	13.76 ± 0.82	0.37 ± 0.10	1.34 ± 0.17	1.24 ± 0.18	1.26 ± 0.06	1.26 ± 0.52	8.85 ± 0.25
K439	M/16	Muzaffarpur/HR	AmB (R)	12.88 ± 0.12	0.77 ± 0.08	1.62 ± 0.134	1.07 ± 0.1	0.87 ± 0.04	4.76 ± 0.20	3.12 ± 0.20
K481	M/32	Muzaffarpur/HR	AmB (R)	17.53 ± 0.34	0.55 ± 0.07	0.69 ± 0.016	2.32 ± 0.14	1.90 ± 0.12	ND	ND
K498	F/55	Madhubani/HR	AmB (R)	15.82 ± 0.24	0.54 ± 0.01	1.16 ± 0.06	1.65 ± 0.05	1.32 ± 0.08	1.38 ± 0.24	7.63 ± 0.24
K516	F/60	Motihari/HR	AmB (R)	16.48 ± 0.61	0.62 ± 0.04	1.26 ± 0.029	2.02 ± 0.10	1.62 ± 0.16	8 ± 0.12	83.33 ± 0.17
K509	F/4	Madhubani/HR	AmB (R)	16.84 ± 0.26	0.65 ± 0.06	ND	2.16 ± 0.13	1.94 ± 0.11	ND	ND
K80	F/40	Bhagalpur/LR	SAG (NR) pentamidine	10.42 ± 2.17	0.18 ± 0.02	0.50 ± 0.006	1.32 ± 0.04	1.58 ± 0.16	1.24 ± 0.48	1.71 ± 0.48
K111	F/36	Siwan/LR	SAG (R)	5.63 ± 0.57	0.2 ± 0.01	1.02 ± 0.07	0.85 ± 0.19	0.47 ± 0.06	3.4 ± 0.12	5.26 ± 0.19
K132	F/24	Munger/LR	ND	3.95 ± 0.28	0.22 ± 0.01	1.22 ± 0.11	0.48 ± 0.05	0.53 ± 0.03	5.5 ± 0.16	55.55 ± 0.16
K133	M/20	West Bengal/LR	SAG (R)	3.45 ± 0.28	0.21 ± 0.01	0.65 ± 0.18	0.93 ± 0.10	0.83 ± 0.04	1.5 ± 0.26	4.52 ± 0.26
K135	F/45	Gopalganj/LR	SAG (R)	4.22 ± 0.38	0.25 ± 0.04	0.31 ± 0.014	0.72 ± 0.03	0.86 ± 0.03	2.86 ± 0.29	1.67 ± 0.29
K216	M/14	West Bengal/LR	SAG (R)	2.14 ± 0.28	0.25 ± 0.03	0.52 ± 0.018	0.91 ± 0.19	0.76 ± 0.02	1.4 ± 0.27	9.43 ± 0.26
K435	M/17	Kushinagar/LR	AmB (R)	11.82 ± 1.39	0.17 ± 0.01	0.57 ± 0.012	1.08 ± 0.25	1.16 ± 0.19	2.67 ± 0.19	4.76 ± 0.21
K59M20		Lab generated, MIL resistant	NA[g]	ND	ND	ND	>15	>15	13.33 ± 0.2	100 ± 0.03
K417M20		Lab generated, MIL resistant	NA	ND	ND	ND	>15	>15	11.3 ± 0.17	100 ± 0.02

[a] M, male; F, female.
[b] Responses were noted 30 days after treatment with SAG infusions (20 mg/kg of body weight) or with AmB infusions (1 mg/kg of body weight) on alternate days for 1 month. Patients with an absence of fever and with a reduction in spleen size were designated responders (R); patients who did not exhibit those outcomes were considered nonresponders (NR).
[c] Mean ED_{50}s ± standard deviations of the results from three separate assays. Amas, amastigotes; Pro, promastigotes.
[d] Expression levels indicative of decreases relative to those seen with strain *Ld*AG83.
[e] 4.28-fold increase relative to the results seen with strain *Ld*AG83.
[f] ND, not determined.
[g] NA, not applicable.

tibility to MIL, with ED_{50} values at the amastigote stage ranging from 0.48 ± 0.05 to 2.32 ± 0.14 μg/ml (mean = 1.38 ± 0.55 μg/ml) and ED_{90} values ranging from 3.53 ± 0.12 to 12.83 ± 1.2 μg/ml (mean = 6.59 ± 3.22 μg/ml). The ED_{50} values for promastigotes (n = 19) ranged from 0.40 ± 0.05 to 1.94 ± 0.11 μg/ml (mean ED_{50} = 1.18 ± 0.11 μg/ml), whereas ED_{90} values ranged from 1.64 ± 0.34 to 8.83 ± 2.67 μg/ml (mean ED_{90} = 3.98 ± 0.87 μg/ml). The antileishmanial activity of MIL revealed a strong correlation between the susceptibility of amastigotes and that of promastigotes (r_{rank} = 0.82). The isolates from HR zones (n = 12) showed a mean ED_{50} of 1.65 ± 0.14 μg/ml, which was significantly higher than that of the isolates from LR region, for which the corresponding value was 0.90 ± 0.1 μg/ml ($P < 0.001$). Overall, we observed a significant positive correlation for the SAG susceptibility profile with AmB (r = 0.599, $P < 0.01$) or MIL (r = 0.66, $P < 0.01$), indicating the possibility of a development of resistance to MIL and AmB. Susceptibility profiles of the field isolates for MIL and AmB were also positively correlated (r = 0.57, $P < 0.01$).

The gene expression levels of LdMT and LdRos3 were determined for VL field isolates (n = 17) in comparison with the expression levels seen with the standard *L. donovani* *Ld*AG83

TABLE 2. Oligonucleotide sequences of genes amplified in this study

Gene	Primer description	Primer sequence (5′→3′)	Product size (bp)
GAPDH[a]	Forward	GAA GTA CAC GGT GGA GGC TG	206
	Reverse	CGC TGA TCA CGA CCT TCT TC	
LdMT[b]	Forward	CAA GTG CCT TTC CAC CAG AAT C	228
	Reverse	CTC ACC TTT TTG AAC TCC AAC AGG	
LdRos3[c]	Forward	ACG ACA CGG CTT GAT TTT CG	238
	Reverse	GAG TAG TCC ACG GAG GCA GTA AAG	

[a] GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
[b] LdMT, *L. donovani* putative miltefosine transporter.
[c] LdRos3, β-subunit of LdMT.

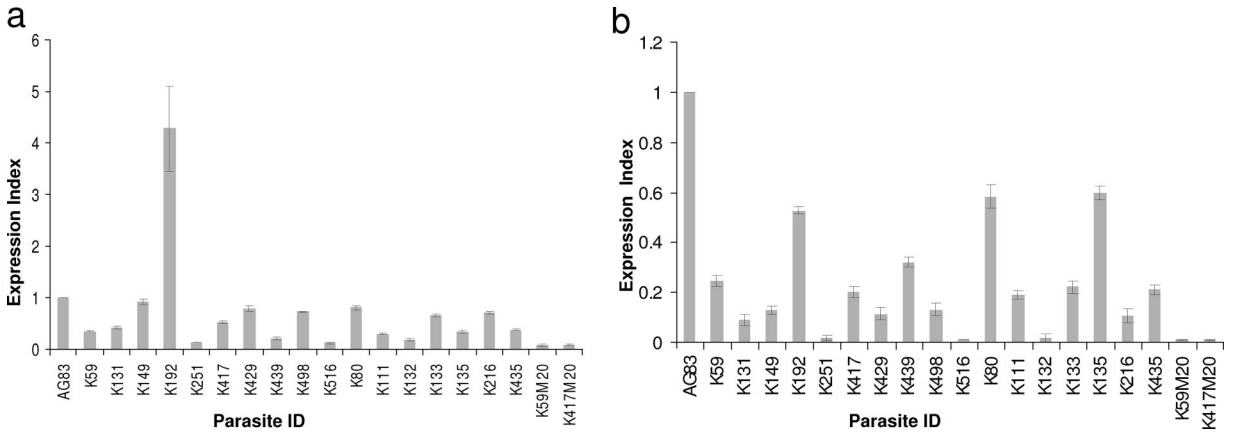

FIG. 1. Expression of LdMT and LdRos3 in different field isolates. (a) Real-time reverse transcription-PCR expression analysis of an *L. donovani* MIL transporter (LdMT). The graph shows the expression index, defined as ratios of gene expression relative to that of strain *Ld*AG83. (b) Real-time reverse transcription-PCR expression analysis of an *L. donovani* MIL transporter (LdRos3). The graph shows the expression index, defined as ratios of gene expression relative to that of strain *Ld*AG83. Data represent the means of the results of three independent experiments.

strain and two MIL-resistant *Ld*M20 parasite strains (generated by a stepwise increase in the concentration of MIL up to 20 μg/ml) by real-time PCR using SYBR green and analyzed by the $2^{-\Delta\Delta Ct}$ method. In comparison to strain *Ld*AG83, the majority of the isolates, except one (K192), revealed decreased expression of LdMT and LdRos3. In general, the levels of expression of LdMT and LdRos3 were correlated and expression of LdRos3 was higher than that of LdMT, though the ratios of expression of LdMT and LdRos3 differed greatly (from 0.67- to-10 fold) among the isolates. In comparison to the results seen with the two laboratory-generated MIL-resistant *Ld*M20 parasite strains, the levels of expression of LdMT and LdRos3 differed more than threefold for the majority (14/17 [82.3%]) of the isolates (Tables 1 and 2 and Fig. 1).

Considering that drug resistance is a manifestation of multifactorial phenomena, various determinants may be responsible for variations in the drug susceptibility of field isolates. Differences in membrane sterol content (1, 5) and lipid content (2) have been demonstrated to lead to distinct drug susceptibility profiles. The extensive use of SAG in areas of hyperendemicity may have changed the biochemical composition of these parasites' membranes in ways that might affect drug susceptibility. Both AmB and MIL are known to interact with the plasma membrane of the cells (5, 13), and membrane modifications have also been suggested as a mechanism of resistance in SAG-resistant isolates (6). Previous studies indicated that some common mechanism of resistance, such as permanent modification in the membranes or drug transporters, etc., may be operating that may modulate drug-induced cell death and may lend cross-resistance to the drugs (6, 7, 14, 21, 22).

The present study highlights the possibility of the occurrence of cross-resistance to three drugs, i.e., SAG, AmB, and MIL, in field isolates, emphasizing the need for novel strategies for treatment of VL. Development of antimony resistance in the anthroponotic VL cycle suggests that resistance to other antileishmanial drugs could also develop once they are widely used as single agents.

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