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Lack of correlation between the promastigote back-transformation assay and miltefosine treatment outcome

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Objectives: Widespread antimony resistance in the Indian subcontinent has enforced a therapy shift in visceral leishmaniasis treatment primarily towards miltefosine and secondarily also towards paromomycin. *In vitro* selection of miltefosine resistance in *Leishmania donovani* turned out to be quite challenging. Although no increase in IC_{50} was detected in the standard intracellular amastigote susceptibility assay, promastigote back-transformation remained positive at high miltefosine concentrations, suggesting a more 'resistant' phenotype. This observation was explored in a large set of Nepalese clinical isolates from miltefosine cure and relapse patients to assess its predictive value for patient treatment outcome.

Methods: The predictive value of the promastigote back-transformation for treatment outcome of a set of Nepalese *L. donovani* field isolates ($n=17$) derived from miltefosine cure and relapse patients was compared with the standard susceptibility assays on promastigotes and intracellular amastigotes.

Results: In-depth phenotypic analysis of the clinical isolates revealed no correlation between the different susceptibility assays, nor any clear link to the actual treatment outcome. In addition, the clinical isolates proved to be phenotypically heterogeneous, as reflected by the large variation in drug susceptibility among the established clones.

Conclusions: This *in vitro* laboratory study shows that miltefosine treatment outcome is not necessarily exclusively linked with the susceptibility profile of pre-treatment isolates, as determined in standard susceptibility assays. The true nature of miltefosine treatment failures still remains ill defined.

Introduction

Since miltefosine was licensed for the treatment of visceral leishmaniasis (VL) in 2002, it largely replaced the pentavalent antimonials within the kala-azar elimination programme in India, Nepal and Bangladesh.[1] However, increasing numbers of miltefosine treatment failures are now being reported, with incidences reaching almost 20%.[2] Although no absolute miltefosine resistance could yet be demonstrated in *Leishmania donovani* relapse isolates,[3] the emergence of resistant strains can indeed be anticipated in view of its pharmacodynamics.[4] At present, actual miltefosine resistance in field isolates has only been documented for a few *Leishmania infantum* isolates.[5,6] We recently developed an *in vitro* resistance selection protocol for intracellular amastigotes, resulting in rapid generation of paromomycin-resistant *L. infantum* and *L. donovani* isolates.[7] While selection of miltefosine resistance could not be achieved, promastigote back-transformation (PBT) clearly suggested selection towards decreased susceptibility upon successive selection cycles.[5] The present study aimed to validate this observation in a set of 17 *L. donovani* isolates from miltefosine responders and relapses from endemic regions in Nepal. To evaluate susceptibility fluctuations within a population after drug exposure,[7,8] two relapse and two cure isolates were cloned and the susceptibility of the established clones was determined.

Materials and methods

Strain selection and parasite culture

The isolates were provided by the Institute of Tropical Medicine Antwerp (ITMA), Antwerp, Belgium, and isolated from bone marrow aspirates of patients from the Terai endemic region in Nepal (BP Koirala Institute of Health Sciences, Dharan, Nepal) (Table 1). The strains were typed as *L. donovani* by cysteine proteinase B (CPB) PCR–RFLP[9] and their full genome sequences are available (J. C. Dujardin, ITMA, unpublished data; Downing *et al.*[10]). Promastigotes were cultured in haemoflagellate

Table 1. Clinical background and susceptibility profile of the selected Nepalese *L. donovani* patient isolates

	Clinical information		Susceptibility assay		
Strain	miltefosine treatment outcome	isolation pre- or post-treatment	IC_{50}, intracellular amastigotes (μM)	IC_{50}, extracellular promastigotes (μM)	positive PBT (μM)
BPK496/0	**cure**	**pre**	**1.8 ± 0.1**	**8.4 ± 1.7**	**20**
BPK648/0	cure	pre	1.0 ± 0.1	3.2 ± 0.4	10
BPK500/0	cure	pre	2.8 ± 0.3	6.4 ± 0.9	20
BPK501/0	cure	pre	ND		10
BPK602/0	cure	pre	1.9 ± 0.2	5.3 ± 0.9	20
BPK612/0	cure	pre	ND		20
BPK616/0	**cure**	**pre**	**1.2 ± 0.2**	**9.2 ± 4.1**	**20**
BPK491/0	cure	pre	2.7 ± 0.3	5.3 ± 0.5	5
BPK499/0	cure	pre	1.8 ± 0.1	6.1 ± 1.6	20
BPK411/0	relapse	pre	1.9 ± 0.1	3.1 ± 0.2	1.25
BPK450/18	relapse	post	3.7 ± 0.5	5.3 ± 1.1	20
BPK513/0	**relapse**	**post**	**2.6 ± 0.2**	**11.8 ± 0.7**	**10**
BPK514/0	**relapse**	**pre**	**2.9 ± 0.3**	**8.9 ± 2.2**	**20**
BPK519/0	relapse	pre	1.1 ± 0.1	2.6 ± 0.9	10
BPK648/2	relapse	post	1.2 ± 0.3	2.6 ± 0.8	10
BPK475/9	relapse	post	1.4 ± 0.1	5.3 ± 0.6	10
BPK652/6	relapse	post	2.2 ± 0.2	12.6 ± 0.7	20
BPK275/0 cl18	control		5.8 ± 1.0	2.4 ± 0.3	5
LEM5159	control		>20	>40	20

The left-hand side shows clinical outcome after miltefosine treatment and time of isolation. On the right, susceptibility values (IC_{50}) for miltefosine (μM ± SEM) based on the standard intracellular amastigote and promastigote susceptibility assays are presented, in addition to the highest miltefosine concentration at which PBT became positive.
A number of strains were cloned (BPK496/0, BPK513/0, BPK514/0 and BPK616/0) to evaluate their polyclonal nature (highlighted in bold; results are presented in Table 2).
ND, not done.

modified Eagle's medium (HOMEM) supplemented with 10% inactivated FCS. The miltefosine-susceptible *L. donovani* reference strain MHOM/NP/03/BPK275/0 cl18 (Kaladrug-R) was included as the negative control, while *L. infantum* MHOM/FR/96/LEM5159, isolated from a French HIV patient with a miltefosine-resistant phenotype at both the promastigote and amastigote levels, was included as the positive control.[5]

Susceptibility determination

To determine the resistance phenotype upon miltefosine exposure, amastigote and promastigote susceptibility assays and PBT assays were performed as previously described.[5] Briefly, extracellular promastigotes and intracellular amastigotes in primary mouse peritoneal macrophages were exposed to 2-fold miltefosine dilutions before determining their IC_{50} values, respectively, with resazurin and by light microscopy after Giemsa staining. PBT was evaluated by mechanically releasing residual intracellular amastigotes after treatment and allowing subsequent promastigote expansion in HOMEM at room temperature. Miltefosine (Sigma Diegem, Belgium) was serially diluted in demineralized water. The lowest in-test concentration was 0.97 μM. The susceptibility values are based on at least three independent repeats, each with two replicates. The cut-off IC_{50} value for resistance was set at 15 μM.[11]

Cloning

To evaluate the composition of promastigote populations and assess individual promastigote susceptibility, two miltefosine relapse isolates and two miltefosine cure isolates were cloned as previously described[7] and susceptibility to miltefosine was determined for each established clone (Table 2). The BPK275/0 cl18 Kaladrug reference, which had undergone several miltefosine selection cycles, was used as a control.[5]

Statistical analysis

The difference between the cure and relapse samples was evaluated using a one-way ANOVA. Results were considered statistically significant if $P < 0.05$.

Results

Results of the standard amastigote and promastigote susceptibility assays and the PBT assay are summarized in Table 1. No statistical differences were observed between the cure and relapse isolates based on the standard intracellular assay after Giemsa staining ($P > 0.05$). In addition, no significant differences could be observed between the relapse and cure group based on the PBT assay ($P > 0.05$). The miltefosine-resistant reference strain LEM5159 showed IC_{50} values of >20 μM upon Giemsa staining with logically also a positive PBT, while for the susceptible reference strain BPK275/0 cl18 (IC_{50} 2.4 ± 0.3 μM), PBT was positive at 5 μM. Cloning efficacy varied considerably among the selected strains (BPK496/0, BPK513/0, BPK514/0 and BPK616/0) (Table 2) and promastigote susceptibility revealed significant dissimilarity between clones of the same isolate, which was not at all reflected at intracellular amastigote level. No significant correlations were found between the different assays.

Table 2. Miltefosine susceptibility profile of the different clones

Strain	BPK275/0 cl18 MIL (laboratory-selected strain)			BPK616/0 (MIL, cure)			BPK513/0 (MIL, relapse)			BPK514/0 (MIL, relapse)			BPK496/0 (MIL, cure)		
	am IC_{50}	prom IC_{50}	PBT	am IC_{50}	prom IC_{50}	PBT	am IC_{50}	prom IC_{50}	PBT	am IC_{50}	prom IC_{50}	PBT	am IC_{50}	prom IC_{50}	PBT
WT	1.4±0.4	2.9±0.9	20	1.2±0.2	9.2±4.1	20	2.6±0.2	11.8±0.7	10	3.0±0.3	8.9±2.1	20	1.8±0.1	8.4±1.7	20
cl1	1.7±0.3	13.5±0.8	20.0	>0.94	3.3±0.6	17.3	2.7±0.2	4.5±1.8	15	4.0±0.4	10.9±1.5	20.0	2.1±0.2	2.9±0.2	15.0
cl2	1.6±0.3	1.1±0.0	20.0	3.1±0.5	16.6±2.4	20.0	2.9±0.3	3.4±0.1	20	6.6±0.2	13.8±0.7	20.0			
cl3	2.1±0.4	0.3±0.0	20.0	>0.94	6.0±1.2	13.1	2.9±0.1	2.9±1.1	11.4	4.4±0.5	14.1±0.0	20.0			
cl4	2.2±0.2	0.6±0.1	20.0	3.1±0.3	7.3±1.2	18	2.5±0.2	5.8±1.7	6.6						
cl5	2.5±0.2	5.5±1.9	20.0	1.0±0.0	11.1±0.9	20	1.2±0.1	3.7±2.0	1.9						
cl6	3.0±0.2	3.6±1.3	20.0	1.2±0.1	29.8±1.5	12.2	1.3±0.1	3.7±0.1	13.1						
cl7	1.7±0.2	0.6±0.1	20.0	>0.94	7.1±0.0	11.4	2.7±0.3	7.4±1.3	15.0						
cl8	2.4±0.3	0.5±0.1	15.0	>0.94	9.1±0.8	11.4	2.1±0.1	7.3±0.1	18.2						
cl9	1.1±0.1	9.1±1.4	20.0	2.8±0.1	7.0±0.0	18	1.3±0.1	14.1±0.1	4.3						
cl10	3.3±0.1	5.3±2.2	20.0	2.7±0.3	20.1±3.3	7.5	3.6±0.3	8.2±0.4	5						
cl11	1.5±0.2	3.1±0.8	20.0	1.0±0.0	7.0±0.1	6.9	1.5±0.1	11.1±0.4	1.3						
cl12				1.1±0.2	23.8±0.9	20	2.6±0.2	8.5±0.7	20						

MIL, miltefosine.
IC_{50} values (μM; mean±SEM) of intracellular amastigotes based on standard Giemsa staining (am) (left) are presented in addition to promastigote susceptibility values (prom) (middle) and the highest miltefosine concentration resulting in a positive PBT assay (right).
Cloning was performed on the BPK275/0 cl18 reference strain, which had undergone eight subsequent cycles of miltefosine treatment (BPK275/0 cl18 MIL)[5] and on the selected miltefosine relapse and cure isolates (Table 1).
The cut-off IC_{50} value for miltefosine resistance was set at 15 μM.[11]

Discussion

Since the kala-azar elimination programme was implemented in 2005, miltefosine has largely replaced pentavalent antimony therapy as first-line treatment in the Indian subcontinent.[1] However, while no widespread miltefosine resistance has yet been observed in the field, concerns about the sustained efficacy of miltefosine as first-line drug have arisen, related to the increasing incidence of treatment failures.[2,12–14] Although decreased miltefosine susceptibility has been observed in Brazilian relapse isolates[15] and full resistance in two French *L. infantum* relapse isolates,[5,6] no miltefosine-resistant phenotype could yet be demonstrated in relapse *L. donovani* isolates based on standard susceptibility assays.[3,13] The predictive value of promastigote assays for assessment of intracellular susceptibility remains highly debatable. On the other hand, one must also recognize that routine application of intracellular amastigote assays is limited due to the poor adaptation of field isolates to *in vitro* culture conditions, as well as their high cost and laborious and time-consuming character. Hence, novel assays that will enable monitoring of miltefosine susceptibility and predict treatment outcome are still awaited. A pilot study on a few *L. donovani* strains suggested the possible application of the PBT assay as a method that could correlate with miltefosine treatment outcome.[5] The present study specifically aimed to validate this hypothesis on a larger sample set of miltefosine cure and relapse patient isolates.

No statistical differences could be demonstrated between the cure and relapse samples, based on either the PBT assay or the standard susceptibility assays on promastigotes and intracellular amastigotes. Although the intracellular amastigotes demonstrated susceptibility to miltefosine within normal ranges (<5 μM),[11] PBT still became positive at concentrations up to 20 μM, which prompted us to check for a possibly polyclonal nature since the presence of individual parasites with a more resistant phenotype could explain why PBT becomes positive at higher concentrations.[7,8] The cloning efficiency of two relapse and two cure isolates (BPK496/0, BPK513/0, BPK514/0 and BPK616/0) proved to be quite variable (Table 2), with significant variability in miltefosine drug susceptibility between individual clones of the same population, but without any relevant difference between the cure and relapse isolates. Phenotypic heterogeneity was also observed among clones derived from BPK275/0 cl18 MIL, a parental cloned strain submitted experimentally to several cycles of miltefosine pressure. A similar observation was made by Coelho *et al.*,[16] who reported that miltefosine resistance is heterogeneous at the level of the population, with individual clones differing in terms of genotype and phenotype. While the use of promastigotes has been suggested for routine miltefosine susceptibility monitoring,[17] the observed differences between promastigote- and amastigote-based susceptibility results favours the use of intracellular amastigotes to assess drug susceptibility, despite the fact that predictions regarding treatment outcome remain invalid.[18] Since current results do not explain the increasing numbers of miltefosine treatment failures,[13] other factors may be involved, such as the involvement of host immunity, parasite virulence[2] and the well-established association between inadequate drug exposure and relapse probability.[19,20]

In conclusion, the PBT assay shows inadequate predictive value for miltefosine treatment outcome. In addition, no correlation

could be found between the time at which PBT is first observed to be positive and the initial number of infecting parasites (S. Hendrickx, unpublished data), although a quantitative role for *in vitro* screening purposes has been proposed.[21] As suggested by Coelho *et al*.,[16] analysis of drug resistance in several clones might be recommendable as it appears to reveal more information than population analysis. However, if in the future VL control were to become dependent on *in vitro* predictions of miltefosine treatment outcome, a quest for other assays enabling distinction between response and relapse would become pivotal. Although it is quite reassuring that the development of primary miltefosine resistance might proceed less straightforwardly than originally anticipated, the increasing treatment failures in the Indian subcontinent cannot be neglected and certainly warrant continued epidemiological monitoring.

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Transparency declarations

None to declare.

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