

Evaluation of anti-leishmanial activity of artemisinin combined with amphotericin B or miltefosine in *Leishmania donovani* promastigotes

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ABSTRACT

Background: The increasing incidence of drug resistance in Leishmaniasis necessitates evaluation of combination chemotherapy. Miltefosine and amphotericin B are established anti-leishmanial drugs, while artemisinin has shown significant leishmanicidal activity in experimental models. In this study, we have evaluated the additive/synergistic effect of artemisinin with amphotericin B or miltefosine.

Methods: *Leishmania* parasites were isolated from the bone marrow aspirate of a patient with visceral leishmaniasis. Parasites were typed as *Leishmania donovani* by restriction fragment length polymorphism of internal transcribed spacer 1 region of *Leishmania* genome. Promastigotes were incubated in a fixed ratio combination of artemisinin (0-500 µM) and amphotericin B (0-100 nM) or miltefosine (0-100 µM) and cell viability was assessed. An isobologram was constructed to evaluate the additive/synergistic effect, wherein it was considered additive if the mean sum fractional inhibitory concentration (mean ΣFIC) at the IC₅₀ level was <2, but ≥1 and synergism, if the mean ΣFIC was <1.

Results: The isobologram showed an additive effect for three combinations of artemisinin-amphotericin B and artemisinin-miltefosine, the mean ΣFICs ranging from 1.02 to 1.44 and 1.08 to 1.33 along with a synergistic effect with one combination, the mean ΣFICs being 0.58 and 0.81 respectively.

Conclusions: This study supports the combination use of artemisinin-amphotericin B and artemisinin-miltefosine, worthy of future pharmacological consideration.

Keywords: Antileishmanial activity, Artemisinin, Amphotericin B, Miltefosine, Promastigotes

INTRODUCTION

Leishmaniasis is a vector borne disease caused by the protozoan parasite *Leishmania sp.* mainly manifested as cutaneous, mucocutaneous and visceral leishmaniasis (VL); in addition, a few patients in the Indian subcontinent and East Africa upon recovery from VL develop Post Kala-Azar dermal leishmaniasis. About 350 million people are at risk of developing the disease in 88 countries world wide¹ with India, Bangladesh, Brazil, Nepal, and Sudan accounting for 90% cases of VL.²

Sodium stibogluconate (SSG) remains the first line treatment against VL all over the world; however, in India, the increasing incidence of unresponsiveness to SSG has limited its use.^{3,4} The newer drugs, include amphotericin B and its liposomal preparations, which though less toxic, is expensive. Miltefosine, initially developed as an anti-cancer drug, is orally effective, but its teratogenicity, low

therapeutic index and potential for developing resistance pose limitations.⁵ Taken together, the current treatment options are far from adequate and the need for combination chemotherapy has been tested in the laboratory⁶ and clinical setting with encouraging outcomes where they have proved efficacious and safe, thereby increasing patient compliance and reducing emergence of drug-resistant parasites, important considerations for the treatment of VL in the Indian subcontinent.⁷

Artemisinin, a sesquiterpene lactone, obtained from plant *Artemisia sp.*, is an established anti-malarial drug used mainly for falciparum malaria.⁸ At present, artemisinin based combination therapy involving either artesunate-mefloquine or artemether-lumefantrine is recommended for treatment of uncomplicated falciparum malaria to prevent recrudescence as well as emergence of drug resistance.⁹ Artemisinin has shown significant anti-leishmanial activity in experimental models¹⁰⁻¹² and considering its effectiveness

in combination chemotherapy in malaria, we evaluated the *in vitro* additive/synergistic effect of artemisinin combined with amphotericin B or miltefosine against *Leishmania* promastigotes.

METHODS

Reagents

All chemicals were obtained from Sigma Aldrich Chemicals (St. Louis, MO, USA) except phenazine methosulfate (PMS, Sisco Research Laboratories, Mumbai, India), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS), inner salt (Promega, Madison, WI, USA). Artemisinin and amphotericin B were dissolved in dimethyl sulfoxide to prepare 100 mM and 1 mM stock solutions, respectively. Miltefosine was freshly prepared in M199 medium (2 mM).

Parasite isolation and transformation

Leishmania parasites (amastigotes) were isolated from a bone marrow aspirate of a patient with VL. The aspirated material was collected and diluted with Schneider's insect medium 1:1, supplemented with 20% heat inactivated fetal calf serum, penicillin G (50 IU/ml) and streptomycin (50 µg/ml) in a tissue culture flask (25 cm²). After incubation at 24°C, culture growth was evident after 6 days.¹³ After transformation from amastigotes to promastigotes, they were gradually adapted into M199 supplemented with 10% fetal calf serum, penicillin G (50 IU/ml), and streptomycin (50 µg/ml) and subcultured every 72 hrs, inoculum being 1 × 10⁶/ml.

Species specific typing of *L. donovani* strains

The parasites were typed as *L. donovani* using restriction fragment length polymorphism (RFLP) of the amplified internal transcribed spacer 1 (ITS1) region. DNA was isolated from *L. donovani* promastigotes by QIAamp DNA mini kit (Qiagen, Hilden, Germany) and eluted in 200 µl elution buffer. The ITS1 region was amplified using primers LITSR (5'-CTGGATCATTTTCCGATG-3') and L5.8S (5'-TGATACCACTTATCGCACTT-3'). Amplification reactions were performed in 25 µl reaction volume and PCR conditions followed as previously described.¹⁴ The negative and positive control was distilled water and DNA of a World Health Organization reference *L. donovani* (MHOM/IN/1980/DD8) strain respectively. The amplified ITS1 region was digested using *Hae III* (Fermentas, Glen Burnie, MD, USA); briefly, reactions were carried out using 1U of *Hae III*, 1X buffer, 5 µl of the amplicon (approximately 100 µg of DNA) and incubated at 37°C for 3 hrs. The digested product was analyzed by electrophoresis (3% agarose, 5 V/cm for 1.5 hrs) along with a GeneRuler™ low range DNA ladder (Fermentas, Glen Burnie, MD, USA) and visualized

in G-BOX gel doc (Syngene, Cambridge, UK) using Gene Tools Software (version 4.01.04, La Jolla, CA, USA).

Drug combinations

The dilutions of artemisinin, amphotericin B and miltefosine were prepared in fixed ratios (Tables 1a and 1b), wherein the starting ratio of artemisinin (µM):amphotericin B (nM) was 0:100, 100:80, 200:60, 300:40, 400:20, and 500:0, respectively and for artemisinin (µM):miltefosine (µM) was 0:100, 100:80, 200:60, 300:40, 400:20, and 500:0, respectively (solutions 1-6). The solutions were then serially diluted (Tables 1a and 1b) and cell viability assay was performed.

Evaluation of anti-promastigote activity

The anti-leishmanial activity of artemisinin, miltefosine and amphotericin B was established in promastigotes, cell viability being measured by the MTS assay.¹⁵ Briefly, log phase promastigotes (1 × 10⁵ cells/200 µl/well) were incubated with artemisinin (0-500 µM), amphotericin B (0-100 nM), miltefosine (0-100 µM) alone and in combination (Tables 1a and 1b) for 48 hrs at 24°C. At the end of 48 hrs, 20 µl of a solution comprising MTS (2.0 mg/ml) and PMS (0.92 mg/ml) in a ratio of 5:1 was added per well. The plates were then incubated further for 3 hrs at 37°C and absorbances of the resultant formazan measured at 490 nm using a plate reader (BioRad, California, USA). MTS is converted to formazan by mitochondrial dehydrogenases of viable parasites in presence of an electron coupler PMS. Therefore, the amount of formazan produced and thereby the intensity of color change was considered to be a measure of cell viability. The mean percent viability was calculated as:

$$\frac{\text{Mean specific absorbance of drug treated parasites}}{\text{Mean specific absorbance of untreated parasites}} \times 100$$

Specific absorbances were determined by subtracting the background absorbance of medium. For each combination (1-6), its inhibitory concentration 50 (IC₅₀) i.e. the concentration that inhibited 50% cell growth, was enumerated by graphical extrapolation using GraphPad Prism software (version 4.01.04, La Jolla, CA, USA). From the IC₅₀ obtained of each combination, its fractional inhibitory concentration (FIC) was derived using the following formula:¹⁶⁻¹⁸

$$\text{FIC} = \frac{\text{Concentration of drug in combination to produce IC}_{50}}{\text{Concentration of drug alone required to produce IC}_{50}}$$

Preparation of isobologram

An isobologram was constructed with mean FIC to determine the interactions between drug artemisinin and amphotericin B or artemisinin and miltefosine. The sum FIC (ΣFIC) value

Table 1a: Ratio of artemisinin (μM) and amphotericin B (nM).

Combinations	1	2	3	4	5	6
Serial dilutions	0:100	100:80	200:60	300:40	400:20	500:0
	0:50	50:40	100:30	150:20	200:10	250:0
	0:25	25:20	50:15	75:10	100:5	125:0
	0:12.5	12.5:10	25:7.5	37.5:5	50:2.5	62.5:0
	0:6.25	6.25:5	12.5:3.75	18.75:2.5	25:1.25	31.25:0
	0:3.12	3.12:2.5	6.25:1.87	9.37:1.25	12.5:0.62	15.62:0

Table 1b: Ratio of artemisinin (μM) and miltefosine (μM).

Combinations	1	2	3	4	5	6
Serial dilutions	0:100	100:80	200:60	300:40	400:20	500:0
	0:50	50:40	100:30	150:20	200:10	250:0
	0:25	25:20	50:15	75:10	100:5	125:0
	0:12.5	12.5:10	25:7.5	37.5:5	50:2.5	62.5:0
	0:6.25	6.25:5	12.5:3.75	18.75:2.5	25:1.25	31.25:0
	0:3.12	3.12:2.5	6.25:1.87	9.37:1.25	12.5:0.62	15.62:0

for each of the six combinations was determined to classify the drug-drug interaction¹⁶⁻¹⁸ as $\Sigma\text{FIC}=\text{FIC}(\text{artemisinin})+\text{FIC}(\text{amphotericin B})$ or $\text{FIC}(\text{miltefosine})$ where $\Sigma\text{FIC} < 1$ represented synergism, $\Sigma\text{FIC} \geq 1$, but < 2 represented additive interaction and $\Sigma\text{FIC} \geq 2$ indicated antagonism.

RESULTS

The parasite isolate showed a ITS1 PCR-RFLP pattern similar to the reference strain MHOM/IN/80/DD8 and accordingly was typed as *L. donovani*. The IC_{50} of artemisinin, amphotericin B and miltefosine were 108.2 μM , 18.75 nM and 18.67 μM respectively, corresponding to previous studies.^{10,15}

Regarding the combination study, two sets of five dose response curves were obtained from each replicate of the combination assay (Tables 1a and 1b) with each set representing four combination solutions and one curve for a drug alone (Figures 1 and 2). Combination dose-response curves were prepared to obtain IC_{50} of each drug in combination. Extrapolating IC_{50} of one drug in combination, it has been assumed that the other drug has not contributed to the anti-leishmanial effect and vice versa, and the FIC_{50} of each combination was accordingly calculated. The mean FIC_{50} values were plotted for six drug combinations to obtain an isobologram of each combination experiment (Figure 3).

Interaction between artemisinin and amphotericin B

The mean FIC of each combination was calculated at different fixed ratios and the isobologram was constructed. Combination five showed synergism ($\Sigma\text{FIC} < 1$), while combinations 2, 3 and 4 showed additive interactions ($\Sigma\text{FIC} < 2$ but ≥ 1). No antagonistic interaction was found

(Table 2a). Figure 3a shows the graphical representation of the interaction.

Interaction between artemisinin and miltefosine

The isobologram prepared from the fixed ratio values showed synergism between the two drugs in combination 5 ($\Sigma\text{FIC} < 1$), whereas combinations 2, 3, 4 showed additive interactions ($\Sigma\text{FIC} < 2$ but ≥ 1). No antagonistic interaction was found (Table 2b, Figure 3b).

DISCUSSION

Artemisinin (Chinese-qinghaosu), a sesquiterpene lactone, is an established anti-malarial drug, but has also shown good leishmanicidal activity in experimental models.¹¹ Amphotericin B and miltefosine both are known anti-leishmanial drugs, but have dose limiting toxicity; more over miltefosine is susceptible to rapid development of resistance owing to its long half-life.⁵

Artemisinin and miltefosine have been reported to generate free radicals within the *Leishmania* parasites, which possibly contributes toward its leishmanicidal activity.^{10,11,19} Amphotericin B inhibits ergosterol biosynthesis; *Leishmania* parasites possess a high ergosterol content and intracellular accumulation through aqueous pores causes their cell lysis.⁴

Artemisinin has a shorter duration of action when compared to miltefosine or amphotericin B, so the combination may be valuable in ameliorating emergence of drug resistance as recommended in treatment of falciparum malaria.

Taken together, this study supports the combinatorial use of artemisinin-amphotericin B or artemisinin-miltefosine

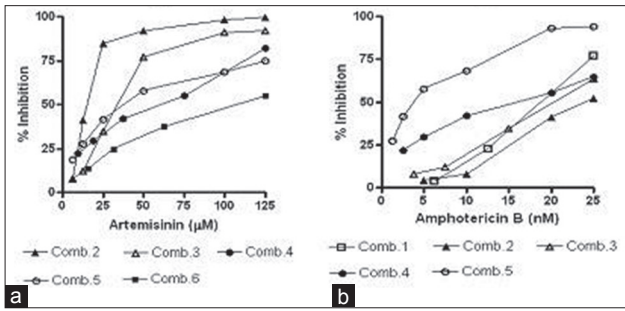


Figure 1: Drug concentration-response curves of artemisinin (a) and amphotericin B (b) in different combinations of artemisinin-amphotericin B (each point represents the mean of at least three experiments in duplicate).

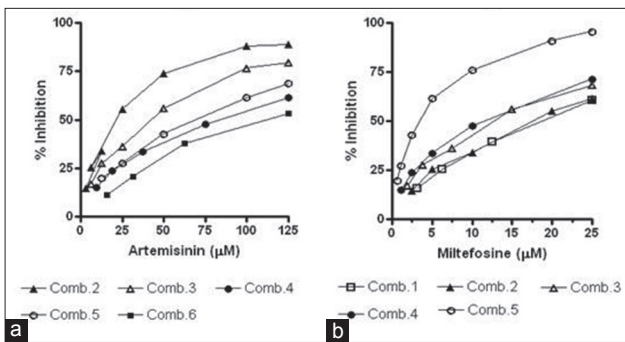


Figure 2: Drug concentration-response curves of artemisinin (a) and miltefosine (b) in different combinations of artemisinin-miltefosine (each point represents the mean of at least three experiments in duplicate).

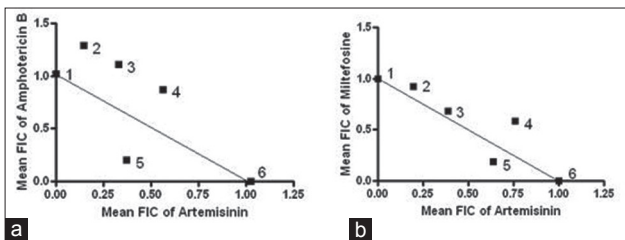


Figure 3: Isobolograms showing interaction between artemisinin-amphotericin B (a) and artemisinin-miltefosine (b). The straight line joins both axes at combination one and six of the mean fractional inhibitory concentration values.

in VL, and understandably should be validated *in vivo*. These combinations may have special relevance in the Indian subcontinent, where drug resistance is a significant problem and the therapeutic armamentarium to date is limited.

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Table 2a: Interaction between artemisinin and amphotericin B against *L. donovani* promastigotes.

Combination	*Mean FIC ₅₀ ±SD		Mean ΣFIC
	Artemisinin	Amphotericin B	
1	0.0	1.023±0.038	1.023
2	0.147±0.012	1.293±0.127	1.440
3	0.332±0.017	1.110±0.046	1.442
4	0.563±0.025	0.873±0.025	1.436
5	0.374±0.004	0.207±0.006	0.581
6	1.023±0.034	0.0	1.023

*Promastigotes (1×10⁵/200 µl/well) were incubated with artemisinin in combination with amphotericin B as described in materials and methods. Each value is the mean of at least three experiments in duplicate. SD: Standard deviation, FIC: Fractional inhibitory concentration

Table 2b: Interaction between artemisinin and miltefosine against *L. donovani* promastigotes.

Combination	*Mean FIC ₅₀ ±SD		Mean ΣFIC
	Artemisinin	Miltefosine	
1	0.0	1.002±0.053	1.002
2	0.197±0.021	0.927±0.074	1.124
3	0.389±0.048	0.687±0.072	1.076
4	0.757±0.038	0.591±0.030	1.348
5	0.631±0.025	0.183±0.006	0.814
6	1.001±0.036	0.0	1.001

*Promastigotes (1×10⁵/200 µl/well) were incubated with artemisinin in combination with miltefosine as described in Materials and methods. Each value is the mean of at least three experiments in duplicate. SD: Standard deviation, FIC: Fractional inhibitory concentration

Conflict of interest: None declared

Ethical approval: Ethical clearance was taken from Institutional Ethics Committee prior to initiation of the study

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