Novel Agents against Miltefosine-Unresponsive Leishmania donovani

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Visceral leishmaniasis is a deadly endemic disease. Unresponsiveness to the only available oral drug miltefosine poses a big challenge for the chemotherapy of the disease. We report a novel molecule, PS-203 [4-(4,4,8-trimethyl-7-oxo-3-oxabicyclo[3.3.1]non-2-yl)-benzoic acid methyl ester], as effective against a miltefosine-unresponsive strain of the parasite. Further, combinations of PS-203 with miltefosine were also evaluated and showed promising results against a miltefosine-unresponsive strain.

Available drugs against leishmaniasis exhibit high toxicity and do not prevent emergence of drug resistance. Miltefosine unresponsiveness is a major concern in India (1). Combinations of available drugs need to be considered apart from the discovery of novel drug candidates effective against prevalent drug-unresponsive strains (2). Combinations of imiquimod and meglumine antimonite for treatment of cutaneous leishmaniasis reduced the amount of meglumine antimonite needed, the time of treatment, and the occurrence of drug resistance due to antimony-based therapy (3). Further, a combination of miltefosine and paromomycin proved to be the most cost-effective treatment strategy in India in recent times (4). Several other combinations of the drugs were reported to be more efficient (5–10). However, not many attempts have been made to discover new drugs for killing miltefosine-unresponsive strains.

The failure of miltefosine, the only available oral drug, is a big threat, especially in India (1). Miltefosine transporter protein LdMT, and, more specifically, its beta subunit LdRo3, is involved in the formation of miltefosine translocation machinery (7, 11). A single point mutation in the LdMT is reported to be responsible for miltefosine resistance in Leishmania (7, 11). However, there seem to be several other factors responsible for miltefosine unresponsiveness. We have earlier reported that miltefosine-unresponsive strains are better able to resist reactive oxygen species (ROS) (12). Thus, to tackle miltefosine-unresponsive strains, much better ROS-producing drug candidates are required.

Our group previously reported the oxabicyclo derivative PS-203 [4-(4,4,8-trimethyl-7-oxo-3-oxabicyclo[3.3.1]non-2-yl)-benzoic acid methyl ester] as a good antileishmanial agent with low toxicity in vitro and in vivo (13, 14). PS-203 disturbs the redox homeostasis of the parasite. In this report, we evaluated the efficacy of PS-203 against a miltefosine-unresponsive strain in vitro. Further, combinations of PS-203 with other antileishmanial compounds were tested against the miltefosine-unresponsive strain.

Parasites, cell lines, and chemicals. The Leishmania donovani (MHOM/IN/10/BHU1081) and miltefosine-unresponsive Leishmania donovani (MHOM/IN/10/BHU1155) strains were obtained from Shyam Sundar (Banaras Hindu University, India) and were cultivated in an M199 liquid medium supplemented with 15% heat-inactivated fetal bovine serum (FBS), 100 U ml⁻¹ penicillin, and 100 µg ml⁻¹ streptomycin. BHU-1155 is a miltefosine-unresponsive isolate that was obtained from the splenic biopsy specimen of a patient after a month of miltefosine treatment and was also used in our earlier studies (12). The human macrophage cell line U937, which was used in this study, was taken from the National Centre For Cell Science (NCCS), Pune, India, and was cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U ml⁻¹ penicillin, and 100 µg ml⁻¹ streptomycin. All of the chemicals used in the experiments were procured from Sigma-Aldrich or Merck. PS-203 was synthesized in our laboratory (15).

Antileishmanial activity assay on promastigote cells and viability assay on human macrophage cells. Antileishmanial effects on promastigote cells and the viability of human macrophage cells were investigated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, and the 50% inhibitory concentration (IC₅₀) was calculated as described earlier (14, 16).

Antileishmanial activity assay on amastigote cells. The human macrophage cell line U937 was made adherent by using 100 ng/ml phorbol 12-myristate 13-acetate (PMA) treatment overnight (17). Macrophages were then infected with miltefosine-unresponsive promastigotes for 48 h. Different concentrations of PS-203 were added and further incubated for 24 h. After incubation, cells were fixed in methanol and were Giemsa stained. Antileishmanial effect of PS-203 toward the intracellular amastigotes was evaluated by microscopic counting of 100 infected macrophage cells and was compared with an untreated control. The parasite density in treated cells was expressed as a percentage of the control.

Effect of PS-203 in combination with miltefosine was evaluated for the miltefosine-unresponsive promastigote strain. The fractional inhibitory concentration (FIC) index was calculated by the following formula: FIC index = [A]/IC₅₀A + [B]/IC₅₀B, where IC₅₀A and IC₅₀B are the IC₅₀s of miltefosine and PS-203, respectively, when tested alone, and [A] and [B] are the IC₅₀s of the miltefosine or PS-203 when treatment was carried out in combination. An FIC index of ≤0.5 indicates synergy while an index of >4 indicates antagonism, and an index in a range between 0.5 and 4 indicates indifference (18, 19).

The miltefosine-responsive and miltefosine-unresponsive strains were grown in our lab, and sensitivity toward miltefosine was confirmed by MTT assay. The data show a biphasic
death pattern in the killing of miltefosine-unresponsive Leishmania donovani (BHU-1155) upon treatment with miltefosine, and the IC50 was found to be higher than that for the wild-type strain, which is 100 μM (Fig. 1A). The data reconfirm that the strain is miltefosine unresponsive in laboratory conditions. The effect of PS-203 on miltefosine-unresponsive Leishmania donovani (BHU-1155) as well as miltefosine-responsive Leishmania donovani (BHU-1081) was also evaluated in similar laboratory conditions for comparison (Fig. 1B). The data indicate that PS-203 is equally effective against the two strains of miltefosine, with an IC50 of 4.0 ± 0.5 μM. The IC50 of PS-203 against the miltefosine-responsive strain observed was marginally lower than our previous report (20).

We also explored a combination of PS-203 with miltefosine in

FIG 1 Effect of miltefosine or PS-203 on Leishmania promastigotes. (A) Miltefosine treatment for a miltefosine-unresponsive strain of L. donovani promastigote Ld1155. The IC50 for the Ld1155 strain is 100 μM. The data confirms that the Ld1155 strain is a miltefosine-unresponsive strain. (B) Effect of the PS-203 compound on Ld1081 and Ld1155 strains of L. donovani. The data indicate that the compound is equally effective on the two strains. The IC50 of PS-203 against the MHOM/IN/10/BHU1081 and MHOM/IN/10/BHU1155 strains is 4.0 ± 0.5 μM, which is marginally low compared to our earlier studies (14). The chemical structure of the PS-203 compound [4-(4,4,8-trimethyl-7-oxo-3-oxabicyclo[3.3.1]non-2-yl-benzoic acid methyl ester] is shown in the inset of panel B. Data represent the mean ± standard deviation (SD) of the results of three independent experiments.

FIG 2 Effect of the miltefosine and PS-203 combination on the miltefosine-unresponsive strain of L. donovani promastigote (BHU 1155) as well as on the miltefosine-responsive strain of L. donovani promastigote (BHU 1081). (A) PS-203 shows significantly improved efficacy in the presence of miltefosine against the miltefosine-responsive strain. (B) The miltefosine-unresponsive strain treated with PS-203 alone and with PS-203 (2.5 μM) and miltefosine (10 μM, 50 μM, or 100 μM) in combination. The combination of the drugs has shown improved efficacy. Data represent the mean ± SD of the results of three independent experiments.
the miltefosine-responsive and miltefosine-unresponsive strains of *Leishmania* (Fig. 2). The data show that PS-203 shows improved efficacy in combination with miltefosine. The IC$_{50}$ of PS-203 was $>1 \mu M$ with 25 $\mu M$ miltefosine for the miltefosine-responsive strain (Fig. 2A). Interestingly, as shown in Fig. 2A, 25 $\mu M$ miltefosine without PS-203 (0 $\mu M$ on the x axis) can kill only 50% of *Leishmania* promastigotes, but in the presence of 1 $\mu M$ PS-203, its efficacy is significantly higher. Similarly, combination of PS-203 with miltefosine decreased the viability of miltefosine-unresponsive promastigotes. The data shown in Fig. 2B were used for the calculation of the FIC index obtained for the miltefosine and PS-203 combination. The combination of PS-203 (1 $\mu M$) and miltefosine (100 $\mu M$) kills 50% of miltefosine-unresponsive promastigotes. The IC$_{50}$ of PS-203 in the presence of 100 $\mu M$ miltefosine was 1 $\mu M$. Likewise, the IC$_{50}$ of miltefosine in the presence of 1 $\mu M$ PS-203 was found to be 100 $\mu M$ (Fig. 2B). The calculated FIC index for the miltefosine-unresponsive promastigotes was found to be 1.25, suggesting that the combination shows an additive response. Similarly, the FIC index for miltefosine-responsive promastigotes was calculated to be $\sim 0.8$, suggesting the combination was additive.

The effects of PS-203 on the amastigote stage of the parasite inside human macrophages and on the antileishmanial activity were studied microscopically. A significant decrease was observed in parasite burden in infected macrophage cells treated with PS-203. Nearly 50% of the amastigotes were inhibited at 5.7 $\mu g$/ml of PS-203 treatment (Fig. 3). Infected macrophages without PS-203 treatment were used as a control. There is a decrease in the number of amastigotes with an increased dose of PS-203 treatment that suggests an antiproliferative effect on amastigotes.

In our earlier studies, PS-203 was found to be safe in an *in vivo* model (13). Further, our current studies show no effect of PS-203 on human macrophage cells (cell line U937) even at higher concentrations up to 100 $\mu M$.

Control of the worldwide spread of leishmaniasis is challenged by the emergence of drug resistance, which is a matter of concern. Miltefosine (hexadecylphosphocholine) was a major breakthrough for the treatment of leishmaniasis. Miltefosine unresponsiveness is a greater challenge in the management of the disease. We have identified the PS-203 compound as very effective against a miltefosine-unresponsive strain. Further, our data suggest that a combination of PS-203 and miltefosine may enhance the efficacy of miltefosine against unresponsive strains as well as responsive strains of *Leishmania donovani*.

**ACKNOWLEDGMENTS**

Research fellowships awarded to M.D. and G.S. by IIT Guwahati are acknowledged. Financial support by the Department of Biotechnology, government of India, in the form of research grants (project no. BT/01/IYBA/2009 and BT/PR3409/MED/29/326/2011) to V.K.D. is also acknowledged.

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