Combination of paromomycin and miltefosine promotes TLR4-dependent induction of antileishmanial immune response in vitro

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Objectives: To evaluate the in vitro activity of antileishmanial drugs, paromomycin and miltefosine, to generate Th-1-biased immunomodulation in hosts against intracellular Leishmania donovani.

Methods: In silico protein–ligand interaction and in vitro drug–cell interaction assays were performed. Interaction assays of TLR4-deficient HEK293 cells and HEK293 cells engineered to express either TLR4 or TLR2 with different concentrations of miltefosine and/or paromomycin sulphate were performed for 48 h. Differentially transfected human peripheral blood monocyte-derived macrophages (PBMFs) were treated with the drugs, and nuclear factor (NF)-κB promoter activity was measured using a κB-luciferase reporter construct. PBMFs were infected with L. donovani. Cultures were incubated with miltefosine or paromomycin sulphate over different concentrations, as mono-treatment or combined. The in vitro antileishmanial effect of the drugs on macrophage-bound L. donovani amastigotes was measured in terms of parasite killing and production of tumour necrosis factor-α (TNF-α) and nitric oxide.

Results: Computational studies reveal that paromomycin and miltefosine interact with TLR4. Both drugs, as mono-therapy or in combination, induce release of TNF-α and nitric oxide in a TLR4-dependent manner. Interestingly, the TLR4-dependent action of the drugs leads to NF-κB promoter activation through MyD88. In vitro, both the drugs kill macrophage-bound L. donovani by inducing release of TNF-α and nitric oxide in a TLR4-dependent manner.

Conclusions: The in vitro activity of paromomycin and miltefosine against host cells is TLR4 dependent. This has implications for: (i) evaluation of in vitro activity of combinational antileishmanial therapy; (ii) the evaluation of drug susceptibility of clinical isolates; and (iii) the standardization of in vitro antileishmanial assays for determining toxicity in hosts.

Keywords: TLR4–drug interaction, combination therapy, TNF-α, nitric oxide

Introduction

Leishmaniasis is one of the neglected tropical diseases and a major global threat for health (http://www.who.int/trad/diseases-topics/leishmaniasis/en/). Visceral leishmaniasis (VL), caused by Leishmania donovani, generally affects resource-poor populations in Bihar, India. Despite many options, treatment of VL in India is far from satisfactory due to increases in drug resistance, relapse and cost. Both paromomycin and miltefosine are proven to be safe, efficient and affordable monotherapeutic options in India, with cure rates >90%.[2,3] However, we have recently demonstrated that their short-course combination is the best treatment option for VL, with a 98.7% (CI: 95.1–99.8) success rate.[4]

Previous studies on the recognition of microbial pathogen-associated molecular patterns (PAMPs) had highlighted the central role of Toll-like receptors (TLRs) in the host defence.[5] Reports suggest that drugs could act as PAMPs to induce cell activation through TLRs, and in turn activate signalling pathways that result in secretion of cytokines, which could mediate immunomodulation.[6] We have recently reported the role of TLR4 re-programming in the development of Th2-biased immune response to promote disease progression in VL.[7] Therefore, stimulating TLR4 by antileishmanial drugs could re-establish an appropriate Th-1-biased immune response leading to cure. However, the molecular basis of action of paromomycin and miltefosine on host macrophages is uncertain. Therefore, we aimed to investigate whether the combination of paromomycin and miltefosine can stimulate TLR4 to induce Th-1-biased immunomodulation.
Methods

In silico protein–ligand interaction
The crystal structure of human TLR4 (accession number: GE-224510867; PDB ID: 3FXI_A [crystal structure of the human TLR4–human MD2–Escherichia coli lipopolysaccharide (LPS) Ra complex]) was downloaded from the Protein Data Bank (PDB). The structures of miltefosine (CID3599) and paromomycin (CID16580) were downloaded from PubChem. Computational protein–ligand interaction studies (human TLR4 with paromomycin and miltefosine) were performed using Discovera studio (DS) software v2.5.

Parasites and cell lines
The parasite used in the study was L. donovani (MHOM/IN/83/AG83). Axenic cultures of the promastigote stage of the parasite were maintained at 22°C as described previously.7 Wild-type HEK293 cells were obtained from NCCS, Pune, India, and cultured according to ATCC instructions. HEK293 cells stably transfected with TLR4 (293–hTLR4A cells) and TLR2 (293–hTLR2 cells) were purchased from InvivoGen, USA.

Patients
Ten randomly selected, diagnostically confirmed VL patients (age: 25.63 ± 7.53; 6 male, 4 female) were enrolled in the study with informed consent as per standard guidelines. This study was approved by the Institutional Ethics Committee of the Rajendra Memorial Research Institute of Medical Sciences, Patna, India. They were treated with a combination of paromomycin and miltefosine as described previously, and blood was drawn before and after the treatment.

Preparation of human macrophages and infection
Human peripheral blood monocyte-derived macrophages (PBMFs) were obtained from heparinized venous blood from healthy volunteers and VL patients as described previously.6 L. donovani amastigotes harvested from the spleen of infected male golden hamsters (6 weeks of age) were used to infect human macrophages at an amastigote:macrophage ratio of 10:1 on chambered slides (Nunc, Denmark) as described previously.7 After incubating cultures overnight at 37°C and 5% CO2 in RPMI 1640 medium plus 10% heat-inactivated autologous serum, non-ingested amastigotes were washed off and drug dilutions added as described below.

Drug–cell interaction assay
Stock solutions (10 mM each) of antileishmanial drugs miltefosine and paromomycin sulphate (Sigma–Aldrich, USA) were freshly prepared in deionized water and filter sterilized. All subsequent dilutions were prepared in RPMI 1640 with 10% heat-inactivated autologous serum on the day of the assay. To correct for potential endotoxin contamination, 10 μg/mL polymyxin B was added in indicated experiments.

Infected macrophages were incubated with drug dilutions for a total of 5 days at 37°C and 5% CO2; medium was changed once after 48 h. The culture supernatant 48 h post-infection was used for a tumour necrosis factor-α (TNF-α) assay and nitric oxide determination. Untreated macrophages received medium plus 10% heat-inactivated (56°C for 30 min) fetal bovine serum (FBS; Sigma–Aldrich, USA) and infection was determined on day 5 post-infection. The 5 day post-infection points also served as untreated controls. In indicated experiments, some cells were pre-treated with anti-human TLR2 monoclonal antibody (50 μg/mL; clone TL2.1), anti-human TLR4 monoclonal antibody (20 μg/mL; clone HTA125) or IgG2a isotype control (all from eBioscience, USA).

Evaluation of drug activity against infected macrophages
Evaluation of drug activity was done at endpoints of the interaction assay; the cells were fixed, Giemsa-stained and analysed for parasite load by microscopy as described previously.6

Flow cytometry
The phenotypical analysis of PBMFs was performed using standardized flow cytometry protocols as described previously.7 All antibodies for immune phenotyping (anti-human CD14, TLR2, TLR4 and relevant isotypes) were purchased from BD Biosciences, USA. Cells were gated according to their forward- and side-scattering patterns. For each marker, 106 cells were counted in the gate.

Transient transfection of cells and measurement of luciferase activity
Transient transfection of HEK293, HEK293-TLR2 and HEK293-TLR4 with plasmids containing nuclear factor (NF)-κB reporter luciferase, CD14, MD2 and MyD88DN (gifts from Dr Fabio Re, University of Tennessee, USA and Professor Jürg Tschopp, University of Lausanne, Switzerland) was performed using FuGENE6 (Roche) transfection reagent following the manufacturer’s instructions. Briefly, cells were incubated with plasmids containing 1 μg of NF-κB reporter luciferase plasmid and, to correct for differences in transfection efficiency, each group of cells was transfected with 80 ng of pSV40/LACZ and co-transfected with 1 μg of MD2/CD14, 3 μg of MyD88DN or 3 μg of empty vector and incubated overnight. The ratio of luciferase activity to β-galactosidase activity in each sample served as a measure of normalized luciferase activity. The cell extracts were prepared for determination of luciferase activity on a luminometer by enhanced luciferase assay reagents (Analytical Luminescence Laboratory) according to the manufacturer’s instructions.

ELISA
Human TNF-α was measured in 48 h post-infection culture supernatant using BD OptEIA kits (BD Biosciences, USA) according to the manufacturer’s instructions.

Measurement of nitric oxide accumulation
The nitric oxide levels in the 48 h post-infection culture supernatant were estimated by reducing the nitrate accumulated over 48 h to nitrite with nitrate reductase, and measuring the nitrite concentration colorimetrically with Griess reagent (Sigma–Aldrich, USA) by a standard method.

Statistical analysis
All experiments were conducted at least in triplicate, and the results are expressed as mean ± SEM. The Mann–Whitney U-test was used to determine statistical significance (GraphPad Prism software v5.0).

Results
Paromomycin and miltefosine interact with TLR4
To investigate whether paromomycin and miltefosine interact with human TLR4 on the cell surface, we first performed computational protein–ligand interaction studies using DS software v2.5; this revealed highest LigandFit scores of 61.236 and 71.039 for TLR4–paromomycin and TLR4–miltefosine interactions, respectively (Figure 1a). Notably, the Lys230 residue of
Interaction of human TLR4 with paromomycin and miltefosine. (a) The GRASP model of human TLR4 (grey) showing interaction with miltefosine (black; upper panel) and paromomycin (black; lower panel). Amino acids Asp181, Ser207, Asp209, Leu208 and Lys230 of TLR4 are found to be frequently involved in interaction (H-bonding) with paromomycin. Likewise, amino acids Lys230 and Arg234 of TLR4 are found to be frequently involved in interaction (H-bonding) with miltefosine. Hence Lys230 is found to interact in both cases (paromomycin and miltefosine). This figure was generated using DS version 2.5. (b) Changes in TLR4 surface expression of macrophages of control healthy subjects with stimulations by various concentrations of paromomycin (P) or miltefosine (M) (combined or as monotherapy) are shown at day 0 [VL-before treatment (VL-BT)] and day 15 [VL-after treatment (VL-AT)]. Differential modulations of TLR4 expressions with respect to stimulations and are expressed as mean fluorescence intensity (MFI; arbitrary units). An asterisk denotes $P<0.05$ (Mann–Whitney U-test). (c) Changes in TLR4 surface expression on macrophages of VL patients before and after chemotherapy with a combination of paromomycin and miltefosine are shown at day 0 (VL-BT) and day 15 (VL-AT). Differential modulations of TLR4 expressions with respect to treatment are expressed as mean fluorescence intensity (MFI). The geometric mean values of MFI of TLR4 in CD14-positive cell populations were determined per donor and compared between VL-BT ($n=10$) and VL-AT ($n=10$). Horizontal bars denote the median values. An asterisk denotes $P<0.05$ (Mann–Whitney U-test). (d) TNF-$\alpha$ and (e) nitric oxide (NO) production by TLR4-mediated cellular activation during exposure to different concentrations of paromomycin (P) and miltefosine (M) (combined or as monotherapy). Differentially transfected HEK293 cells were stimulated with increasing doses of the drugs (combined or as monotherapy), LPS (0.01 $\mu$g/mL) or unpurified $\beta$-galactosidase (b-gal) and are the mean $\pm$ SEM of three experiments performed in triplicate. An asterisk denotes $P<0.05$ versus protein control, OVA (Mann–Whitney U-test). (f) Stable transfection of TLR4 in HEK293 (HEK293-TLR4) rendered a concentration-dependent responsiveness, as indicated by TNF-$\alpha$ and NO secretion, to paromomycin and miltefosine. HEK293-TLR4 cells were responsive to LPS and HEK293-TLR2 cells were responsive to unpurified PGN (not shown). An asterisk denotes $P<0.05$ (Mann–Whitney U-test). Paromomycin (P) and miltefosine (M) (alone (f) and combined (g)) dose–responses for NF-$\kappa$B promoter activity. Control human PBMCs were transfected with NF-$\kappa$B reporter luciferase plasmid or empty vector (see the Methods section), preincubated with or without polymyxin B (10 $\mu$g/mL) then stimulated with different doses of paromomycin and/or miltefosine or 0.1 $\mu$g/mL OVA, and incubated for 2 h after which extracts were prepared for determination of luciferase activity. Results represent the ratio of luciferase (Luc) to $\beta$-galactosidase (b-gal) and are the mean $\pm$ SEM of three experiments performed in triplicate. An asterisk denotes $P<0.05$ versus protein control, OVA (Mann–Whitney U-test). (h) MyD88 and MD2 are essential for TLR4-mediated activation of NF-$\kappa$B promoter activity. HEK293-TLR4 cells were transfected with NF-$\kappa$B reporter luciferase plasmid or with pSV40/LACZ plasmid and cotransfected with MD2, CD14, MyD88DN or empty vector (see the Methods section). Cells were preincubated with or without polymyxin B (10 $\mu$g/mL) then stimulated with paromomycin (P) and miltefosine (M) (3 and 30 $\mu$M, respectively), LPS (0.01 $\mu$g/mL) or 0.1 $\mu$g/mL OVA and incubated for 2 h, after which extracts were prepared for determination of luciferase activity. Results represent the ratio of luciferase (Luc) to $\beta$-galactosidase (b-gal) and are the mean $\pm$ SEM of three experiments performed in triplicate. An asterisk denotes $P<0.05$ versus protein control, OVA (Mann–Whitney U-test).
TLR4 actively participated in interactions with both drugs (Figure 1a). Furthermore, quantitative flow cytometric data revealed that paromomycin and miltefosine, either as mono-treatment or combined, considerably increased TLR4 expression in a concentration-dependent manner in PBMFs from healthy controls (Figure 1b) and also in PBMFs from VL patients after completion of the combined chemotherapeutic treatment (Figure 1c).

**Paromomycin and miltefosine induce release of TNF-α and nitric oxide in a TLR4-dependent manner**

Further, to explore whether paromomycin and miltefosine functionally interact with TLR4 expressed on the cell surface, we employed an interaction assay with TLR4-deficient HEK293 cells and HEK293 cells engineered to express either TLR4 or TLR2, with different concentrations of drugs for 48 h. It is important to mention that HEK293 cells lack TLRs yet retain downstream components used for TLR signalling. HEK293 cells transfected to express specific TLRs can be used to assess TLR recognition of potential ligands; these cells secrete cytokines in response to TLR signalling and consequent activation of NF-κB. Our results demonstrated that paromomycin/miltefosine (miltefosine: 3 μM, 1.22 mg/L; paromomycin: 30 μM, 21.41 mg/L) significantly (P=0.0317) induced secretion of TNF-α in HEK293-TLR4 cells in a dose-dependent manner, but not in TLR4-deficient HEK293 or HEK293-TLR2 cells (Figure 1d). At the highest concentrations, the level of TNF-α was 786.2±12.4 pg/mL in HEK293-TLR4 cells, compared with 142.5±5.3 pg/mL in unstimulated controls (Figure 1d). Our results also showed that paromomycin/miltefosine, at these levels, induced nitric oxide production in HEK293-TLR4 cells significantly (P=0.0302), and in a dose-dependent manner, but not in HEK293 or HEK293-TLR2 cells (Figure 1e). However, induction of TNF-α release or nitric oxide production was considerably less when either paromomycin or miltefosine was used as mono-treatment for HEK293-TLR4 cells, compared with their combined effect (Figure 1d and e).

**Paromomycin/miltefosine combination activates NF-κB promoter activity via MyD88**

As paromomycin and miltefosine were able to initiate TLR4 stimulation, we opted to check whether they send the signal through the MyD88/IRAK–NF-κB transduction pathway. To test this, control human PBMFs were treated with paromomycin and miltefosine alone or combined, and then NF-κB promoter activity was measured using a κB-luciferase reporter construct after 2 h of incubation at 37°C. Our results suggested that mono or combined stimulation significantly (paromomycin (30 μM, 21.41 mg/L; P=0.0023), miltefosine (5 μM, 2.03 mg/L; P=0.0012) and combined (miltefosine 30 μM, 21.41 mg/L; paromomycin 3 μM, 1.22 mg/L; P=0.0326)) increased NF-κB promoter activity (Figure 1f and g). OVA protein was used as control and did not up-regulate NF-κB promoter activity significantly above background levels (Figure 1f and g).

To determine the mechanism by which paromomycin and miltefosine activate the signal cascade that results in the activation of NF-κB promoter activity, we stimulated differentially transfected HEK293-TLR4 cells. Our results demonstrated NF-κB promoter activity in HEK293-TLR4 cells was elevated by LPS (0.01 μg/mL), but not by miltefosine/paromomycin (3 and 30 μM, respectively) (Figure 1h). It was quite interesting to note that paromomycin/miltefosine-induced NF-κB promoter activity was only restored in HEK293-TLR4 cells transiently transfected with both CD14 and MD2 to the level comparable to LPS-induced NF-κB promoter activation, but transfection of CD14 alone did not have any effect (Figure 1h). Therefore, this suggests that TLR4–CD14–MD2 association is essential for paromomycin/miltefosine-mediated NF-κB promoter activation. We further investigated the role of MyD88 in paromomycin/miltefosine-induced NF-κB promoter activation in HEK293-TLR4 cells. Our experiments showed that transient transfection of HEK293-TLR4 cells with the dominant negative MyD88 (MyD88DN—a construct encoding a dominant negative MyD88 with a deletion of its death domain to evaluate roles of MyD88) almost completely abrogated both paromomycin/miltefosine-induced and LPS-induced NF-κB promoter activity, even in the presence of MD2 (Figure 1h). To rule out the role of endotoxin contamination, we preincubated HEK293-TLR4 cells with polymyxin B (10 μg/ml) to abrogate LPS-induced, but not paromomycin/miltefosine-induced NF-κB promoter activity (Figure 1h).

**Paromomycin/miltefosine combination induces parasite killing in L. donovani-infected macrophages in a TLR4-dependent manner**

As paromomycin/miltefosine induced TLR4-dependent Th-1-biased immunomodulation, we aimed to investigate the effect on macrophage-bound L. donovani amastigotes in vitro. Infected human PBMFs were treated with paromomycin and miltefosine (alone or combined), with or without pretreatment with anti-human TLR2/TLR4 monoclonal antibody or IgG2a isotype control. The results showed that neutralization of TLR4, but not of TLR2, significantly (P<0.05) inhibited the anti-leishmanial effect of paromomycin and miltefosine on infected macrophages (Figure 2a). We subsequently investigated the effect of TLR2/4 neutralization on the release of TNF-α and nitric oxide mediated by paromomycin and miltefosine. Quantitative results indicated that neutralization of TLR4, but not of TLR2, abrogated the effects of paromomycin and miltefosine on release of TNF-α (Figure 2b) and nitric oxide (Figure 2c) from L. donovani-infected macrophages.

**Discussion**

The epidemiological scenario of VL makes its elimination a realistic goal. Major advances have been made by the recent introduction of single-dose liposomal amphotericin B and multidrug combinational therapy. A debate on monotherapy versus combination therapy is warranted in context of the development of drug resistance in VL cases in Bihar. As therapy directly targeting TLRs for Th-1-biased immunomodulation would be a good option for elimination of VL in the Indian state of Bihar, it was tempting to investigate which antileishmanial drug or combination can perform such host-cell-modifying functions. Taken together, our results implicate TLR4 in the pro-inflammatory functions of paromomycin/miltefosine in VL. The ensuing TLR4–paromomycin/miltefosine interaction activates...
intracellular signalling pathways that result in nitric oxide and TNF-α secretion, essential for parasite killing. Combination of miltefosine with paromomycin was also reported to be favourable in vivo. Therefore, we propose that selective targeting of TLR4 through combination of paromomycin and miltefosine leads to parasite killing in VL. Our observations show not only the mechanism of the immunomodulatory action of miltefosine with paromomycin for the first time, but also indicate the superiority of the drugs to other antileishmanials by directly targeting TLRs. This study also expands the concept that ‘non-immunological’ drugs can serve as agonists for TLR, consequent activation of which mediates additional beneficial or detrimental drug effects. On the one hand these drugs can be used to target TLR4 for Th-1-biased immunomodulation for parasite killing in VL; on the other hand excess dosage of these drugs can lead to adverse toxic effects as reported for amphotericin B.

The strongest argument for combinations would be to counter the potential of drug resistance in India, paromomycin and miltefosine combination being a promising candidate. However, the advantages of combination therapy have to be weighed against potential disadvantages, as combinations of drugs could increase the severity of adverse effects, especially combined toxicity in vivo. Using a combination of paromomycin and miltefosine as a model treatment for VL, we show for the first time the host TLR-targeting functions of an antileishmanial therapy. Further studies are required to evaluate the potency of other antileishmanials as TLR-targeted host-cell modulators for parasite killing and cure.

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Transparency declarations
None to declare.

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