IJP: Drugs and Drug Resistance 7 (2017) 370–377

Contents lists available at ScienceDirect

IJP: Drugs and Drug Resistance

journal homepage: www.elsevier.com/locate/ijpddr

Transcriptome profiling identifies genes/pathways associated with experimental resistance to paromomycin in *Leishmania donovani*

Aditya Verma[a,b], Vasundhra Bhandari[a,1], Deepak Kumar Deep[a], Shyam Sundar[c], Jean Claude Dujardin[d], Ruchi Singh[a], Poonam Salotra[a,*]

[a] ICMR-National Institute of Pathology, Safdarjung Hospital Campus, New Delhi, India
[b] Symbiosis School of Biomedical Sciences, Symbiosis International University, Pune, India
[c] Institute of Medical Sciences, Banaras Hindu University, Varanasi, India
[d] Unit of Molecular Parasitology, Department of Parasitology, Institute of Tropical Medicine, Antwerp, Belgium

ARTICLE INFO

Keywords:
Leishmania donovani
Drug resistance
Paromomycin
Transcriptome
ABC transporters
Nitric oxide
Visceral leishmaniasis

ABSTRACT

Widespread resistance towards antimony and reports of relapses following miltefosine treatment has severely affected the management of visceral leishmaniasis (VL) in the Indian subcontinent. Paromomycin (PMM), an aminoglycoside antibiotic, has been licensed for VL treatment in India in 2007. Although its use is still restricted in the field, unraveling the molecular mechanism of resistance towards PMM is the key to preserve the drug. In this study, PMM resistant lines were selected up to 100 μM of PMM in three distinct field isolates of *Leishmania donovani* at promastigote stage. The resistance induced at promastigote level was also evident in amastigotes which showed 6 fold decreases in PMM susceptibility. Comparative transcriptome profiling of PMM resistant (PMM-R) and the corresponding PMM sensitive (PMM-S) parasites revealed modulated expression of 500 genes (1.5 fold cut off) in PMM-R parasites. Selected genes were validated for their modulated expression by quantitative real-time PCR. Functional classification and pathway analysis of modulated genes indicated probable adaptations in drug resistant lines which included a) reduced oxidative phosphorylation; b) increased glycosomal succinate fermentation and substrate level phosphorylation; c) dependency on lipids and amino acids for energy generation; d) reduced DNA synthesis and increased DNA damage repair and e) decreased protein synthesis and degradation. Interestingly, PMM-R parasites showed a marked increase in PMM susceptibility in presence of verapamil and amlodipine, antagonists of Ca^{2+} channel that are also modulators of ABC transporters. Moreover, infection of macrophages by PMM-R parasites led to modulated nitric oxide (NO) levels while reactive oxygen species (ROS) level remained unaltered. The present study highlights the putative mechanisms of PMM resistance in *Leishmania*.

1. Introduction

Visceral leishmaniasis (VL) or kala-azar is the most severe form of leishmaniasis and may be fatal, if left untreated. In the absence of any vaccine, control of VL relies mainly on chemotherapy. Existing drugs for VL have serious drawbacks in terms of safety, efficacy, cost, and development of resistance. Miltefosine (MIL) was considered as one of the main pillars of VL elimination program in India; however, reports of relapses following MIL treatment and decline in its efficacy raised concerns regarding its utility in VL control (Bhandari et al., 2012; Sundar et al., 2012; Rijal et al., 2013). In a recent study the declining efficacy of MIL for the treatment of post kala-azar dermal leishmaniasis (PKDL) patients, considered to be a major reservoir for transmission of the disease, has been reported (Ramesh et al., 2015). Another drug, amphotericin B, is associated with toxicity (Srivastava et al., 2011) and its liposomal formulation (AmBisome) has been recommended as a first line treatment of VL in the Indian subcontinent (World Health Organisation, 2010).

Paromomycin (PMM) is another treatment option for VL control in the Indian subcontinent and has been found to be effective as monotherapy as well as in combination with other drugs (Sinha et al., 2011; Sundar et al., 2011; Rahman et al., 2017). Development of resistance towards aminoglycosides such as PMM has been frequently encountered in a variety of bacteria and PMM resistance in *Leishmania* can readily be induced *in vitro* (El-On et al., 1991; Maarouf et al., 1998; Hendrickx et al., 2012). A report of increased ED_{90} towards PMM in

* Corresponding author. National Institute of Pathology (ICMR), Safdarjung Hospital Campus, New Delhi, 110029, India.
E-mail address: salotra@vsnl.com (P. Salotra).
[1] Present Address: School of Life Sciences, University of Hyderabad, Hyderabad, Telangana, India.

http://dx.doi.org/10.1016/j.ijpddr.2017.10.004
Received 7 September 2017; Received in revised form 4 October 2017; Accepted 9 October 2017
Available online 10 October 2017

L.donovani isolates from VL cases residing in a high endemic region is alarming (Prajapati et al., 2012), suggesting that PMM is at considerable risk for the development of dug resistance. Therefore, this issue needs to be proactively addressed in laboratory studies.

Previous studies on PMM resistance in *Leishmania* parasites suggest that drug resistance is associated with increased membrane fluidity, reduced accumulation of drug (Jhingran et al., 2009; Bhandari et al., 2014), altered mitochondrial energy metabolism (Maarouf et al., 1997) and increased tolerance to the host defence mechanisms (Bhandari et al., 2014). In a proteomic study, a number of proteins showed differential expression in experimental PMM resistant (PMM-R) parasites as compared to the wild type (Chawla et al., 2011).

In the present study, gene expression profiling of experimentally selected PMM-R and the corresponding PMM sensitive (PMM-S) parasites was carried out for identification of genes responsible for possible mechanisms of PMM resistance, using oligonucleotide array representing genomic sequences of *L. infantum* and *L. major* (Leprohon et al., 2009). Differentially modulated genes and pathways that may contribute to PMM resistance were identified. Role of ABC transporters was further explored in PMM resistance using verapamil and amlodipine that are Ca^{2+} channel antagonists and ABC transporter modulators. Production of nitric oxide (NO) and reactive oxygen species (ROS) by PMM-S/PMM-R parasites upon infection to the host macrophages was also studied.

2. Materials and methods

2.1. Culture of parasites

Three distinct clinical isolates of *L. donovani*, were obtained from bone marrow aspirates of VL patient, as described in our previous studies (Bhandari et al., 2014; Deep et al., 2017). One of these isolates (MHOM/IN/2000/K133) was obtained from antimony responsive VL patient reporting to Safdarjung Hospital, New Delhi and the other two isolates (MHOM/IN/09/BHU573/0 and MHOM/IN/09/BHU568/0) were obtained from antimony unresponsive patients that reported to Kala-Azar Medical Research Centre, Muzaffarpur, Bihar.

The study was approved by the Ethics Committee of the Institute of Medical Sciences, Banaras Hindu University (Dean, 2007–08/42, dated 15-05-2008), Varanasi and Institutional Ethics committee of Safdarjung Hospital & VMMC (VMMC/SJH/PROJECT/22-10-2012/7), New Delhi, India. Written informed consent was obtained from patients.

Promastigotes were cultured at 25 °C in M199 medium with 25 mM HEPES (pH7.4) supplemented with 10% FBS, 100 IU penicillin G and 100 μg/ml of streptomycin.

2.2. Selection of PMM resistant parasites

PMM resistance was artificially selected in cloned lines (derived by limited dilution cloning) of all the three isolates. Parasites were gradually exposed to increasing PMM concentration (25–100 μM) at promastigote stage. At each step, parasites were cultured for at least 5–8 passages to attain steady and optimal cell growth. The concentration of drug was increased only when the adapted isolate showed comparable growth kinetics to wild type culture as described previously (Bhandari et al., 2014).

2.3. Drug susceptibility assay at promastigote stage

Drug susceptibility assays at promastigote stage were performed as previously described (Deep et al., 2017). Briefly, log-phase promastigotes were harvested and counted in a counting chamber. Promastigotes were seeded into 96-well plates at 5×10^5 parasites/well and incubated with 200 μl medium alone or serial dilutions of PMM (1.9 μM–2000 μM). 50 μl resazurin (Sigma Aldrich, USA) [0.0125% (w/v) in PBS] was added to each well after72-h incubation at 25 °C and the plates were incubated further for 18 h. Cell viability was measured fluorimetrically (λ_{ex} 550 nm; λ_{em} 590 nm, Infinite M200, Tecan, Switzerland). The results were expressed as the percentage reduction in the parasite viability compared to that in untreated control wells. 50% inhibitory concentration (IC_{50}) and 90% inhibitory concentration (IC_{90}) was calculated using sigmoidal regression analysis. All experiments were performed at least thrice in quadruplicates.

2.4. Drug susceptibility assay at intracellular amastigote stage

In vitro PMM susceptibility was assessed at intracellular amastigote level by following macrophage-amastigote model as previously described (Deep et al., 2017). Briefly, the mice peritoneal exudates derived macrophages (PECs) were infected with late log phase promastigotes at a ratio of 10: 1 (parasite: macrophage), plated into 8 well chamber slides and incubated for 16 h at 37 °C in 5% CO_2. Excess, non-adhered promastigotes were removed by washing and infected cells were re-incubated for 48 h with different dilutions of PMM (0, 10, 20, 40, 80, 120 and 150 μM). Macrophages were then examined for intracellular amastigotes after staining with Diff-Quik solutions. The number of *L. donovani* amastigotes was counted in 100 macrophages, at 1000× magnification. The survival rate of parasites relative to untreated macrophages was calculated. IC_{50} and IC_{90} were determined by sigmoidal regression analysis. The assays were performed in duplicate and repeated at least thrice.

2.5. Oligonucleotide array

Single-color microarray-based gene expression profiling was performed using a high-density *Leishmania* multispecies 60-mer oligonucleotide microarray slide [8 × 15K format] representing the entire genome of *L. infantum* and *L. major*. The microarray chip, printed by Agilent Technologies, USA, included a total of 9233 *Leishmania*-specific genes including 540 control probes as described earlier (Rochette et al., 2008; Leprohon et al., 2009; Kulshrestha et al., 2014).

2.6. RNA extraction and evaluation

Total RNA was extracted from 10^8 late log phase promastigotes using Trizol reagent as described by the manufacturer. RNA clean up was performed using RNeasy Plus mini kit (Qiagen, USA) according to manufacturer's instructions. The purified RNA was quantified using Nanodrop by estimating the absorbance at 260 and 280 nm. The quality and integrity of RNA was assessed on RNA 6000 Nano Assay Chips on Bioanalyzer 2100 (Agilent Technologies, USA) (Kulshrestha et al., 2014).

2.7. RNA labelling, amplification, hybridization and data analysis

500 ng of total RNA was reverse transcribed using oligo dT primer tagged to T7 promoter sequence. cDNA thus obtained was converted to cRNA using T7 RNA polymerase enzyme and Cy3 dye. 600 ng of Cy3 labeled cRNA was hybridized on the array (AMADID: 27511) using the Gene Expression Hybridization kit (Agilent Technologies, USA) in Sure hybridization Chambers (Agilent) at 65° C for 16 h. Hybridized slides were washed using Agilent Gene Expression wash buffers (Agilent Technologies, USA) and scanned on a G2505C scanner (Agilent Technologies, USA). Images thus obtained were quantified using Agilent's Feature Extraction Software Version-10.7. Feature extracted raw data was analyzed using GeneSpring GX12.6.1 microarray data and pathway analysis tool. Quartile (75th percentile) normalization was performed. Storey and bootstrapping analysis was performed for multiple testing corrections. All microarray data is available on the GEO NCBI database in the MIAME format; http://www.ncbi.nlm.nih.gov/geo with the GEO accession number GSE74208. DNA microarray data were analyzed by custom R programs to illustrate the expression profile