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ScienceDirect
Molecular & Biochemical Parasitology 150 (2006) 278–287
MOLECULAR & BIOCHEMICAL PARASITOLOGY

The role of *Leishmania enriettii* multidrug resistance protein 1 (LeMDR1) in mediating drug resistance is iron-dependent

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Received 16 June 2006; received in revised form 12 August 2006; accepted 30 August 2006
Available online 18 September 2006

Abstract

In parasitic protozoan *Leishmania enriettii*, the role of a multidrug resistance (*mdr*) gene *LeMDR1* (*L. enriettii* multidrug resistance 1) in mediating vinblastine resistance has been previously demonstrated by association, transfection and "gene knockout" studies. LeMDR1 has been shown to be located intracellularly and it was proposed to mediate drug resistance by sequestering drugs into intracellular organelles rather than by active efflux. Here we compared *LeMDR1* overexpressed cell lines (Vint3 and V160), wild type (Le) and *LeMDR1* "double knockout" mutant (*LeMDR1−/−*) and demonstrated that *LeMDR1* gene copy number was associated with (1) higher level of intracellular iron, (2) increased sensitivity to an iron-dependent antibiotic, streptonigrin and (3) increased enzyme activity of an iron–sulfur-containing mitochondrial enzyme, aconitase. This result suggests that the normal function of LeMDR1 is related to mitochondrial iron homeostasis. To test such hypothesis, we have used the *LeMDR1*-overexpressing mutant V160 and *LeMDR1−/−* mutant to determine how iron level can affect its resistance level to drugs targeting either cytosol (vinblastine) or mitochondria (rhodamine 123 and pentamidine). It was found that the resistance level of V160 to vinblastine can be increased by iron whereas resistance to both rhodamine 123 and pentamidine can be increased by iron depletion and vice versa. Iron treatment can potentiate rhodamine 123 and pentamidine accumulation whereas iron deprivation can cause the reduction of rhodamine 123 accumulation. Our result highly suggests that LeMDR1's function in mediating drug resistance is iron-dependent.

Keywords: *Leishmania enriettii*; Multidrug resistance; *LeMDR1*; Iron transporter; Streptonigrin; Iron(II) sulfate; Bathophenanthroline disulfonic acid

1. Introduction

Numerous drug resistance mechanisms have been identified from *in vitro* studies. One frequent class of resistance mechanisms involves overexpression of membrane proteins belonging to ATP-binding cassette (ABC) transporters that could significantly reduce intracellular drug accumulation by active extrusion of the drug from the cell. In mammalian cells, overexpression of these transporters, including P-glycoproteins (Pgps), the product of the *mdr1* gene [1] and multidrug resistance associated proteins (MRPs) [2] can render both tumor and culture cells highly resistant to many different chemotherapeutic agents.

Leishmaniasis is one of the six major parasitic diseases targeted by World Health Organization (WHO). It is endemic worldwide in 88 countries, especially in the northern Africa, Asia, Mediterranean and Latin America, where it threatens more than 350 million people [3]. Currently, 12 million people are infected with *Leishmania* species and with 1.5–2 million new cases occurring annually [4]. Chemotherapy relies primarily on pentavalent antimony containing drugs and secondarily on pentamidine and amphotericin B. Unresponsiveness to antimonials have been reported since 1940s and this has become a serious public health problem.

In *Leishmania* spp., two different classes of ABC transporters are linked to drug resistance. They exhibit homologies to the mammalian MRP1 (ABCC) and Pgp (ABCB) transporters [5]. First, the *Leishmania* MRP1 (ABCC) homolog, PGPA, has been implicated in metal resistance. The *pgpA* gene is frequently amplified in *L. mexicana* (*LmepgpA*) [6] and *L. tarentolae* (*LtpgpA*) [7] cells selected for resistance to arsenite or antimony

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doi:10.1016/j.molbiopara.2006.08.014

containing drugs. Transfection and disruption experiments indicated that PGPA is indeed involved in metal resistance [8,9].

The second group of ABC transporter in *Leishmania* is the ABCB type transporter (e.g. LeMDR1, LaMDR1, LdMDR1, LtrMDR1), which exhibits a higher sequence similarity to mammalian Pgp at the nucleotide-binding site [10–13]. In *L. enriettii*, the gene *LeMDR1* has been previously demonstrated to be involved in vinblastine resistance by drug-selection, gene-knockout and transfection approaches [11,14,15]. Vinblastine is an inhibitor of tubulin assembly and belongs to a class of MDR substrate. Like mammalian cells, overexpression of LeMDR1 conferring resistance to vinblastine might be through drug efflux. Interestingly, its overexpression was simultaneously associated with increased susceptibility to another MDR substrate, rhodamine 123, which targets mitochondria [14]. It is suggested that LeMDR1 may have a predominantly intracellular localization rather than at the plasma membrane [14]. We have previously proposed that LeMDR1 may mediate vinblastine resistance through sequestering it into subcellular organelle which may connect to the mitochondria, thereby separating vinblastine from its cytosol target: tubulin. In contrast, sequestering rhodamine 123 into this subcellular organelle will have an opposite effect: concentrating rhodamine 123 with its mitochondrial target thereby making the cells hypersensitive to rhodamine 123 [14].

In order to understand how LeMDR1 mediates drug resistance, we have focused on using four strains of isogenic *L. enriettii*, with the only difference being *LeMDR1* gene copy number. They are *LeMDR1−/−*, Le wild type (*LeMDR1+/+*), V160 (*LeMDR1* amplified) and Vint3 (*LeMDR1* transfected) [11,14,15]. *LeMDR1−/−* is a *LeMDR1* double knockout mutant. Le wild type is the wild type strain of *L. enriettii.* V160 is a vinblastine resistant *L. enriettii* obtained by stepwise selection. It contains about 20 copies of extra-chromosomal circle (V-circle) containing *LeMDR1* on it. Vint3 was obtained by transfecting V-circle, tagged with neomycin resistance gene, into *L. enriettii.* The gene copy number of LeMDR1 is in the rank order of *LeMDR1−/−* < Le wild type < V160 < Vint3.

In this report, we will investigate the effect of iron in affecting LeMDR1's role in mediating drug resistance. The reason of studying iron is because: (1) LeMDR1 expression is related to the hypersensitivity to a mitochondrial drug rhodamine 123 thereby suggesting LeMDR1's function is mitochondrial related, (2) there are several mitochondrial Pgps and one of them (ATM1) is related to iron homeostasis in yeast and (3) the enzyme activity of cytochrome *c* oxidase, an iron-containing enzyme, is found to be affected by the LeMDR1 copy number (unpublished).

2. Materials and methods

2.1. Cell lines and cell culture

Promastigotes of *L. enriettii* were cultured in Schneider's *Drosophila* Medium (Invitrogen), pH 6.9, supplemented with 10% (v/v) heat inactivated fetal calf serum (Hyclone) with 4 mM glutamine (Sigma) and 25 μg/mL gentamicin solution (Invitrogen), at 27 °C for 4 days [11]. Parasites were grown for 4 days after each passage until late-log phase was reached (2×10^8 cells/mL). The growth condition of parasite can be observed under inverted microscopy. To pass the cells, 50 μL of late-log phase of parasites were removed to a new 25 cm^2 culture flask. To this flask, 5 mL fresh Schneider's *Drosophila* medium was added and they were allowed to grow for 4 days until next passage.

The *LeMDR1+/+* wild type (Le), *LeMDR1−/−* mutant, *LeMDR1*-overamplified mutant (V160) and V-circle transfectant (Vint3) in which the gene *LeMDR1* is amplified on the V-circle were employed in this study. The *LeMDR1−/−* mutant was grown in the presence of 75 μg/mL of geneticin (Invitrogen) and 200 μg/mL of hygromycin (Invitrogen). The V160 cell line was maintained in 170 μM (160 μg/mL) vinblastine (Sigma) while Vint3 transfectant was maintained in 75 μg/mL of geneticin in order to keep their high copy numbers of V-circle on which the gene *LeMDR1* resides. The Le wild type was grown in drug free Schneider's *Drosophila* medium.

2.2. Total cellular iron (Fe) determination

A 10 mL of promastigotes was used for iron determination and the cells were lysed by repeated freeze–thaw cycles. The iron amount was measured using Graphite Furnace Atomic Absorption Spectrometer. The iron content was normalized to total cellular protein.

2.3. Aconitase enzyme assay

A 50 mL 4-day-old promastigotes was harvested by centrifugation at 3500 rpm for 5 min at room temperature. The pellet was washed twice with PBS pH 7.4 and finally resuspended with 600 μL reaction buffer (50 mM potassium phosphate buffer pH 7.4, 30 mM sodium citrate, 0.5 mM $MnCl_2$). The promastigotes were lysed by repeated freeze–thaw cycles. Then the cell lysate were centrifuged at 16,000 × *g* for 20 min at 4 °C and the supernatant fraction was collected and ready for aconitase assay.

Cell extracts (300 μL) were pipetted to 700 μL solution containing 50 mM Tris–HCl pH 7.4, 30 mM sodium citrate, 0.5 mM $MnCl_2$, 0.2 mM $NADP^+$, 2 units/mL isocitrate dehydrogenase (Sigma). The increase in absorption was measured at 340 nm. A unit of activity was defined as the amount of enzyme which catalyzes the production of NADPH/min, assuming an extinction coefficient of 6.22 mM^{-1} cm^{-1}. Moreover, the enzymatic activity was normalized to total cellular protein. Some controls were set up in which hydrogen peroxide (H_2O_2) and bathophenanthroline disulfonic acid (BPS) (Sigma) were added to the reaction. They are known inhibitors of aconitase enzyme.

2.4. Cell viability assay

The viability of promastigotes was determined by the Cell Titer 96® Aqueous Assay (Promega) that employs a novel tetrazolium compound (MTS) and electron coupling reagent, phenazine methosulfate (PMS). Promastigotes were seeded into 96-well flat bottom microtiter plate at 1×10^5 cells/well in a final volume of 100 μL medium with or without test compounds. The