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Short communication

Intracellular location of the ABC transporter PRP1 related to pentamidine resistance in *Leishmania major*

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Leishmania spp. is the causative agent of leishmaniasis, a parasitic protozoan disease that affects 12 million people. There are around 350 million people exposed to the risk of infection by different species of *Leishmania* [1]. The primary control measure against leishmaniasis is chemotherapy, and pentavalent antimonials are the main drugs used. Amphotericin B and Pentamidine (PEN) are second-line drugs used as alternatives to pentavalent antimony in leishmaniasis treatment [2]. PEN is accumulated by the parasite, and although the primary mode of action is still unclear, its effects include binding and disintegration of kinetoplast DNA [2]. Unfortunately, however, chemotherapy is confronted with ever more frequent cases of resistance. The mitochondrion is an important target of PEN action in kinetoplastids, and a decreasing accumulation of this drug in mitochondria in *Leishmania mexicana* resistant to PEN has been demonstrated [3]. This mechanism of drug resistance appears to be related to reduced mitochondrial membrane potential and is probably caused by a decrease in mitochondrial dehydrogenase and F_1F_0 ATPase activities [3,4]. Studies into the mechanism of drug resistance are thus crucial to allow more rational use of drugs and so minimize or overcome resistance.

In eukaryotic cells, the ABC (ATP-binding cassette) transporter superfamily is made up of highly conserved proteins consisting of two transmembrane domains and two nucleotide binding domains (NBD) that contain the conserved Walkers A and B motifs as well as the ABC transporter signature [5]. In *Leishmania*, 23 ABC transporter genes have been listed in the Genome Project [6], and at least three families of ABC transporters have been described: (1) the ABCA family, whose members are not related to drug resistance [7,8]; (2) the ABCB family that includes MDR1, whose overexpression confers resistance to vinblastine and other hydrophobic drugs [9]; and (3) the ABCC family that includes MRPA (PGPA), a protein related to arsenite and antimonials resistance [10–12].

Recently, we described an ABC transporter termed PRP1 (pentamidine resistance protein 1) that is related to PEN resistance and cross-resistant to trivalent antimonials in *L. major* when overexpressed [13]. As other ABC transporters previously described, the biological role of PRP1 in *Leishmania* metabolism is unclear. PRP1 shares few identity with members of ABC families (less than 40%), indicating that PRP1 is a different ABC transporter belonging to a new ABC family [13]. *PRP1* is a single-copy gene located in chromosome 31 (1500 kb) and corresponds to the first gene identified as being related to PEN resistance in *Leishmania* [13]. Searches in other trypanosomatid (*Trypanosoma brucei* and *Trypanosoma cruzi*) genome databases did not identify a sequence related to PRP1, indicating that this ABC transporter is specific to genus *Leishmania*. Previous analyses of Southern blot data indicated the presence of related PRP1 sequences in other *Leishmania* species (data not shown). In yeast, the product of the *PNT1* gene mediates PEN

Abbreviations: ABC, ATP-binding cassette; GFP, green fluorescent protein; kb, kilobase; MDR, multidrug resistance; MRP, multidrug resistance protein; NBD, nucleotide-binding domain; PCR, polymerase chain reaction; PEN, pentamidine; PRP1, pentamidine resistance protein 1; RT-PCR, reverse transcriptase polymerase chain reaction

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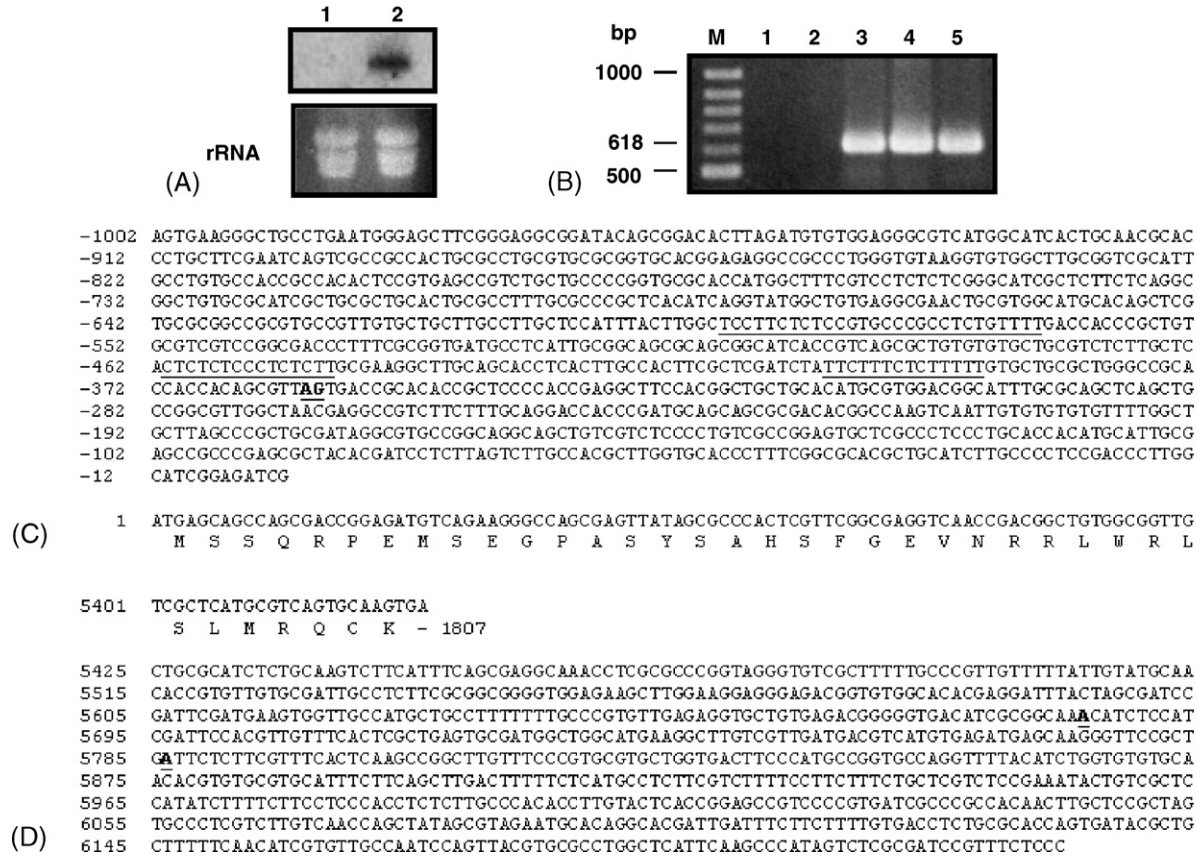


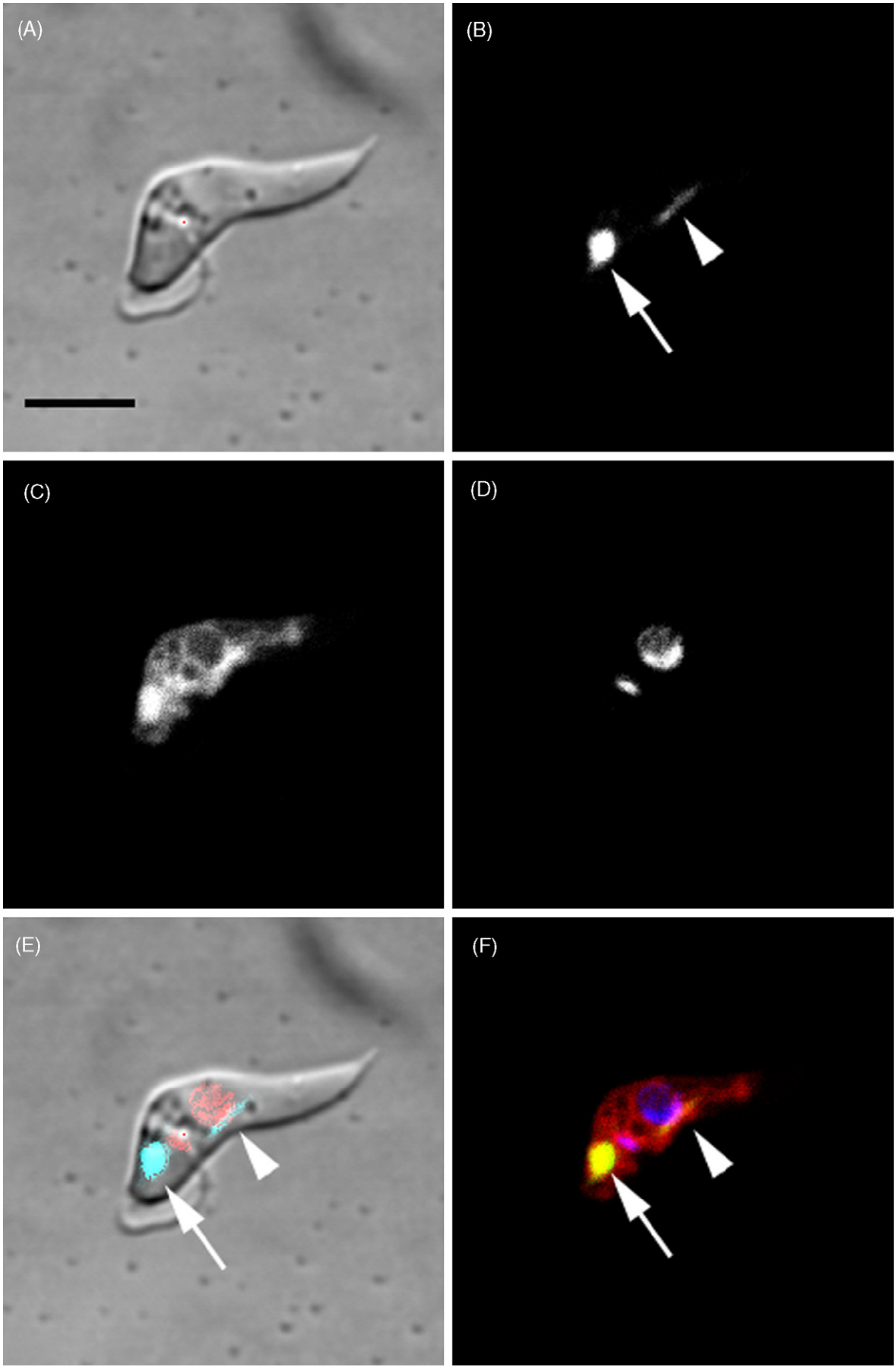
Fig. 1. Analysis of *PRP1* expression. (A) Northern blot analysis of cosPEN1-A and wild-type *L. major*. Total RNA (10 μg) of wild-type *L. major* (1) and transfectant cosPEN1-A (2) were separated by electrophoresis, transferred to a nylon membrane and hybridized [32] with a 0.8 kb *Pst*I fragment corresponding to the NBD1 of PRP1. Ribosomal RNA (rRNA) was used as a loading control. (B) RT-PCR amplification products using the specific primer PRP1R (5′-GCGGTCGACGCCTCATCGCTCTCTAC-3′) for cDNA synthesis, degenerate PRP1 primers NBDIF (5′-GCGGAATTCCYGCGMGSRMRGYTGACRGTKGTG-3′) and NBDIR (5′-GCGGTCGACMGTSGCYAGCACGCGCGTCTTGCC-3′) for PCR and total RNA from wild-type *L. major* promastigotes (3), cosPEN1-A (4) and pSNBR/8 kb *Sma*I-A (5). Reactions without reverse transcriptase confirmed the absence of contaminating DNA in samples (2) and reactions without cDNA were used for PCR control (1). (C) The acceptor-splicing AG dinucleotides at position 358 from the translation-initiation site are in bold and underlined. The polypyrimidine tracts preceding the spliced leader acceptor are underlined. (D) The polyadenylation sites at position +260 and +361 downstream of the stop codon are in bold and underlined.

resistance when overexpressed, and its localization is mitochondrial [14]. Sequence analysis of PNT1 indicates no similarity with ABC transporters, including PRP1 (data not shown).

Cellular localization of ABC transporters in *Leishmania* has recently been described. Members of the ABCA family have been found at both the plasma membrane and flagellar pocket (ABCA1) [7], or restricted to the flagellar pocket, as ABCA2 [8]. Members of ABCB and ABCC families (respectively, MDR1 and MRPA), are located close to the flagellar pocket and are associated with intracellular membranes of structures known to be related to exocytic and endocytic pathways [15,16].

Gene amplification is a common survival mechanism used by *Leishmania* strains selected for resistance to several drugs such as methotrexate, terbinafine, vinblastine and antimonials [9,11,17–19]. In *Leishmania infantum*, amastigotes selected for resistance to potassium antimonyl tartrate [Sb(III)] have the *MRPA* gene amplified as part of an extrachromosomal circle and expressed in higher levels [20]. These recent data show that gene amplification can occur in *Leishmania* amastigotes and mediate drug resistance in the parasite form treated with the drug. Resistance mediated by gene amplification does not, however, appear to be involved in mutant promastigotes resistant to PEN in *Leishmania major* [17]. On the other hand, PRP1 expression does occur in wild-type amastigotes of *Leishmania donovani* that are sensitive to, and those that are resistant to, pentavalent antimonials [21].

With regard to PRP1 expression, we have previously observed by Northern blot analysis that transfectants overexpressing *PRP1* contain transcripts that are not detected in wild-type promastigotes [13]. These data were confirmed using a transfectant containing a 35 kb extension of the *PRP1* locus (cosPEN1A) where a higher transcript (10 kb) was detected in the transfectant, but not in wild-type *L. major* (Fig. 1A). Several authors have shown that detection of *Leishmania* ABC transporter transcripts by Northern blot analysis in wild-type parasites is difficult because of the low levels of expression [7–9,19]. We therefore performed RT-PCR using internal primers of *PRP1* directed to the NBD1 domain of PRP1 and obtained specific products of 618 bp when total RNA from wild-type parasites as well as


(A)
(B)
(C)
(D)
(E)
(F)

different transfectants of *L. major* were used (Fig. 1B). The processing sites of the mRNA of *PRP1* were also detected. Sequencing analysis of the amplified products detected the 5′ and 3′ processing sites of *PRP1* mRNA. Only one trans-splicing site was identified, located 358 nucleotides upstream from the putative translation initiation region of the *PRP1* transcript (Fig. 1C). It was located by reverse transcription using cDNA synthesized from promastigotes and then amplified with a spliced-leader primer and an antisense primer (PRP1 5′1) corresponding to a region 300 nucleotides downstream of the ATG triplet of *PRP1* (data not shown). In addition, two polyadenylation sites were identified using cDNA synthesized with an antisense primer consisting of polyT fused to an Adapter Primer (AP) (Gibco-BRL) and amplified with an Abridged Universal Amplification Primer (AUAP) and an antisense primer corresponding to the 3′ end of *PRP1* (PRP1 3′2). The polyadenylation sites were 260 and 361 nucleotides downstream of the stop codon (Fig. 1D). Our data indicated that the *PRP1* mRNA is 6042 nucleotides long, the coding region contains 5424 nucleotides, the 5′ UTR contains 358 nucleotides and the 3′ UTR is at least 260 nucleotides long.

Expression of PRP1 in wild-type and transfectant parasites was first detected using anti-GST-PRP1 polypeptide antibodies directed to the NBD1 of PRP1. Polyclonal antiserum clearly recognized a single and, expected protein of approximately 180 kDa in transfectants pSNBR/8 kb *Sma*I-B by Western blot. This protein was weakly detected in wild-type parasites, with no signal detected in rabbit preimmune serum (data not shown). Similarly, a low level of MRPA expression was observed in analysis of *Leishmania* wild-type [22]. These data lead us to suggest that despite the low level of *PRP1* gene expression, its presence appears to be sufficient for it to be involved in PEN susceptibility in wild-type promastigote parasites. This hypothesis is corroborated by the fact that Northern blot analysis also failed to detect MDR1 and MRPA transcripts in wild-type promastigotes [9,19], although the respective null mutants showed increased sensitivity to arsenite, antimonite (MRPA) and vinblastine (MDR1) [12,16].

To reinforce that, three different transcripts encoding ABC transporters were also detected in *Trypanosoma brucei* by RT-PCR [23]. Among them, the ABC transporter MRPA, involved in resistance to melarsoprol, had its expression hardly detected in wild-type parasites by Western blot [24]. On the other hand, there are no differences in the expression level of *Trypanosoma cruzi* ABC transporters PGP1 and PGP2 in parasites that are sensitive to or those that are resistant to benznidazole and nifurtimox. Indeed, expression of both genes was detected by Northern and Western blot [25], probably indicating a different mechanism of resistance for these parasites not associated to these ABC transporters.

Alterations in the expression level of genes not directly implicated in amplification were also observed in *Leishmania* mutants resistant to SbIII and methotrexate [26,27]. Similarly, in PEN resistant parasites, higher levels of *PRP1* transcripts and alterations in expression of other unknown genes could be responsible for the resistance phenotype indicating a possible mechanism unrelated to gene amplification, once it was demonstrated that at least in *L. major* gene amplification is not associated with PEN resistance [17]. Another possibility that could involve PRP1 in PEN resistance would be mutations in this ABC transporter that could give an increasing in the transporter activity.

For cellular localization, PRP1 C-terminus was fused with GFP, yielding PRP1-GFP. We first verified that PRP1-GFP fusion was functional and was able to confer a threefold increase in resistance to PEN on transfected promastigotes (plasmid pXG-PRP1-GFP, $IC_{50} = 2.25 \pm 0.3$ μg/ml, compared with wild-type *L. major* $IC_{50} = 0.68 \pm 0.07$ μg/ml), a similar level observed with transfectants containing only PRP1 [13]. Similarly transfectants containing PRP1-GFP fusion also had their PEN resistance phenotype reversed by verapamil (plasmid pXG-PRP1-GFP, $IC_{50} = 2.25 \pm 0.3$ μg/ml, compared with plasmid pXG-PRP1-GFP treated with 15 μM of verapamil $IC_{50} = 0.69 \pm 0.09$ μg/ml) and are cross-resistant to trivalent antimonials (data not shown).

Confocal microscopy analyses of transfected promastigotes imaged live showed that PRP1 is intracellular and located next to the kinetoplast with a large proportion of the GFP signal concentrated within of a distinct intracellular compartment (Fig. 2B, E and F). We also observed that GFP signal was located in a tubular compartment in structure and oriented along the lateral-longitudinal axis of transfected parasites (Fig. 2B, E and F). In view of this, it is probable that PRP1 is located at the tubulovesicular element responsible in part for the exocytic and endocytic

Fig. 2. PRP1 is intracellular in *L. major*. To construct the plasmid pXG-PRP1-GFP, the *PRP1* gene was amplified by PCR using a high fidelity polymerase (Amersham) with the primers PRP1-GFP-F (5′-GCGAGATCTTCGATGAGCAGCCAGCGACCGGAG-3′) and PRP1-GFP-R (5′-GCGCCCGGGA-CACTGACGCATGAGCGACGCGAC-3′). The 5.4 kb product was purified and cloned in the previously digested pXG-′GFP plasmid [33] with *Sma*I. The accuracy of the construct was verified by restriction digest mapping and sequencing of the junction sequence between of PRP1 and GFP genes. Imaging of transfected live cells was carried out by immobilizing parasites in 1% (w/v) low melting point agarose in PBS containing 1% glucose. For fluorescent staining, promastigotes were incubated with 0.1 mM Hoechst 33342 (2,5′-Bi-1H-benzimidazole, 2′-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-trihydrochloride, Molecular Probes, Eugene, Oregon, USA) and LysoTracker (Molecular Probes, Eugene, Oregon, USA) for 30 min. Samples were washed three times in PBS and *L. major* promastigotes were then imaged in a BioRad 1024UV confocal system attached to a Zeiss Axiovert 100 microscope using a 1.4 NA 100× DIC oil immersion PlanApochromatic objective [34]. Images were then processed with Image-J (http://rsb.info.nih.gov/ij/) and Adobe Photoshop. *L. major* transfected with pXG-PRP1-GFP (A–F). (A) Differential interference contrast (DIC) image. Localization of PRP1 was determined by GFP fluorescence that is concentrated close to the kinetoplast (arrow) and along a tubule (arrowhead) (B). Acidic organelles were labeled using LysoTracker (C). Hoechst 33342 was used to stain DNA (both nuclear and kinetoplast) (D). Merged image showing the intracellular location of GFP-PRP1 (cyan) and Hoechst 33342 (red) on the DIC image. PRP1 was also observed in a tubular compartment along the parasite (arrowhead), and next to the kinetoplast (arrow in E). Merged image of B–D showing the localization of acidic organelles (red), nuclear and kinetoplast DNA (blue) and GFP (yellow); the yellow hue (arrowhead and arrow) corresponds to the co-localization of LysoTracker and GFP (F). Bar in A = 5 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

pathways [28,29], as observed for MRPA and MDR1 [15,16]. The acidic compartment corresponding to these tubulovesicular elements were analyzed using Lyso-Tracker-Red™ (Molecular Probes, Eugene, OR) a fluorophore that labels acidic organelles like endosomes and lysosomes. This fluorescent probe was observed along the parasite (Fig. 2F) and in a merged image of GFP and Lyso-Tracker-Red™ a co-localization (yellow) in the tubular structure was observed (Fig. 2F). These data indicated that the tubular compartment containing GFP is acidic in nature. Considering the localization of the yeast *PNT1* gene product to the mitochondrion [14], and the biochemical implication of mitochondrial changes underlying resistance to PEN in *Leishmania* [3,4], it is important to state that our data led us to conclude that PRP1 is not associated with the mitochondrion in *L. major* (Fig. 2E).

We are proposing a mechanism of PEN resistance mediated by PRP1. PEN would be taken up by a high-affinity PEN transporter, probably a proton symporter, similar as described in *L. mexicana* [3]. The ABC transporter PRP1 would confer resistance by sequestering PEN in vesicles that would be exocytosed by the cell throughout the flagellar pocket. It is well known that the flagellar pocket is located at the flagellum base and is related to cellular endocytosis and exocytosis in trypanosomatids [30]. Similarly, MRPA, acting on metal–thiol conjugates, is also associated with intracellular membranes, where sequestration of metals into the vesicles could be exocytosed [15]. Nevertheless, Basselin et al. (2002) showed that in wild-type *L. mexicana* PEN is accumulated into the mitochondrion, while in resistant parasites PEN remains free in cytosol. They also state that an ABC transporter inhibited by verapamil like PRP1 [13], and other compounds like trifluoperazine and prochloperazine would be responsible for removing cytosolic PEN from the cell [3]. It is possible that this efflux could not be directly driven by a plasma membrane ABC transporter, but it could equally involve pumping PEN into a vesicle followed by vesicular efflux. In this hypothesis, PRP1 could have an important role to remove PEN from the cytosol of *Leishmania*. In *L. major* genome, 23 members of the ABC transporter family have been described [6] most of which have not yet been characterized. Further studies are needed to identify these ABC transporters members and elucidate more precisely the mechanism of sensitivity and resistance to PEN.

In *L. donovani* cells raised by step-wise PEN selection, PEN efflux has not been evidenced [4]. In this cells P-glycoprotein mediated efflux of PEN seems to be not operative as it is in *L. mexicana* [3], or in *L. major* over-expressing episomal PRP1, as we are suggesting in this work. However, it is important to point out here that differences at molecular level amongst *Leishmania* species are generally accepted as a reason for the variation in the pharmacological efficacy of anti-*Leishmania* compounds [31].

The role of PEN in *Leishmania* metabolism remains unclear, and PRP1 seems to have an important role in PEN sensitivity/resistance. We have already reported that even in nontoxic concentrations the calcium-channel blocker verapamil is an important inhibitor of PRP1 [13]. We thus propose that this compound could be used experimentally in association with PEN to determine its efficacy against *Leishmania* amastigotes in vitro and in vivo, and also in future as a therapeutic option in patients with leishmaniasis.

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