Characterization of an ABCG-Like Transporter from the Protozoan Parasite Leishmania with a Role in Drug Resistance and Transbilayer Lipid Movement

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Leishmaniasis treatment is hampered by the increased appearance of treatment failure. ATP-binding cassette (ABC) transporters are usually involved in drug resistance both in tumor cells and in microorganisms. Here we report the characterization of an ABCG-like transporter, LiABCG6, localized mainly at the plasma membrane in Leishmania protozoan parasites. When overexpressed, this half-transporter confers significant resistance to the leishmanicidal agents miltefosine and sitamaquine. This resistance phenotype is mediated by a reduction in intracellular drug accumulation. LiABCG6 also reduces the accumulation of short-chain fluorescent phospholipid analogues of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine. As a whole, these results suggest that LiABCG6 could be implicated in phospholipid trafficking and drug resistance.

First-line treatment against visceral leishmaniasis is based on pentavalent antimonials, but their efficacy is compromised by the increased appearance of drug resistance (7, 9). Amphotericin B and miltefosine were recently recommended as alternative therapeutic drugs. Other drugs in clinical trials are paromomycin and the 8-aminoquinoline sitamaquine.

Experimental resistance to miltefosine due to inactivation of the miltefosine transport complex (26, 27), as well as to overexpression of ABC transporters (6, 28, 30), has been described recently. ABC transporters constitute one of the largest families of proteins described, with a broad variety of physiological functions and considerable medical and economical consequences. These proteins are highly evolutionarily conserved and ubiquitous; they are present from prokaryotes to humans (13). ABC transporters use energy from ATP hydrolysis to translocate their substrates (ions, heavy metals, carbohydrates, amino acids, antibiotics, anticancer drugs, proteins, phospholipids, steroids, or pigments) across the cell membrane. The essential structure of an ABC transporter consists of two highly conserved nucleotide binding domains (NBD), which bind and hydrolyze ATP, and two hydrophobic transmembrane domains (TMD), with six membrane-spanning helices. Eukaryotic ABC transporters can be organized either as full-size transporters (two NBD and two TMD) or as half-transporters, containing just one of each domain. Half-transporters are thought to require dimerization in order to assemble a functional protein.

Among the substrates of ABC transporters, many of the aminooquinolines used for malaria treatment (chloroquine, mefloquine, primaquine) can be found. MRP1 (ABCC1) transports both chloroquine and mefloquine (23, 38). P-glycoprotein (MDR1, ABCB1) interacts with mefloquine (3). The Plasmodium falciparum pfmdr transports mefloquine and is involved in the phenomenon of drug resistance (34). On the other hand, many ABC proteins are implicated in the movement of phospholipids and cholesterol among organelles in mammalian and yeast cells (32). ABCA1 transports cholesterol and phosphatidylethanolamine (PC) to ApoA-I; ABCB4 transports PC into the bile (35). Similarly, members of the ABCG subfamily have been associated with lipid transport (18, 31, 35). ABCG transporters are half-transporters, with an NBD-TMD topology; therefore, dimerization is required for them to become functionally active. Mammalian ABCG1 mediates cholesterol, PC, and sphingomyelin efflux (17, 33), and it is thought to heterodimerize with ABCG4 (10). Although mammalian ABCG2 was described primarily as a multidrug transporter, it can also be involved in lipid transport processes. ABCG2 mediates the transport of unconjugated steroids and sulfated conjugates of bile acids and steroids (36). Mammalian ABCG5 and ABCG8, which are expressed to high levels in epithelial cells of the intestine and act as a heterodimer, have been associated with the efflux of phytosterols and cholesterol into bile (11). Recently, ABCG-like proteins required for the secretion of surface waxes have been described in Arabidopsis spp. (4).

The complete sequence of the Leishmania genome (14) has allowed the elaboration of a complete inventory of ABC transporters found in Leishmania spp.—42 transporters belonging to all subfamilies described in eukaryotes (A to H) (20). To date, only ABC transporters related to the ABCA, ABCB, ABCD, and ABCG subfamilies have been described for Leishmania spp. (2, 5, 6, 8, 24, 29). In this work, we report the characterization of the Leishmania infantum ABCG6 gene (LiABCG6), which encodes an ABCG-like transporter localized at the parasite plasma membrane (PM). LiABCG6 has...
phospholipids, alkyl-phospholipids, and aminquinolines as potential substrates and seems to be responsible for their active outward transport from the cytoplasmic to the exoplasmic leaflet of the parasite PM. While this work was in progress, the ABCG6 gene of *Leishmania donovani* was described. ABCG6 is involved in camptothecin resistance due to rapid efflux [5].

**Materials and Methods**

Materials. Miltefosine and perifosine were from Zentaris (Frankfurt, Germany). Edelfosine was purchased from Calbiochem (Darmstadt, Germany). Hexadecylyphospho[1,2-enolpyruvyl-3-phosphatidylcholine ([32P]NBD-PC), -phosphoethanolamine (NBD-PE), and -phosphoserine (NBD-PS) and 6-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]aminohexanoyloxy-glycerol-3-phosphocholine (NBD-PC), -phosphoethanolamine (NBD-PE), and -phosphoserine (NBD-PS) were from Avanti Polar Lipids (Birmingham, AL).

**Cell Cultures.** Promastigotes of *L. infantum* (strain MHOM/ES/1993/BCN-99) were grown in vitro at 28°C in modified RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 20% heat-inactivated fetal bovine serum (Invitrogen). DNA constructs and transformation procedures. *L. infantum* (GenBank accession no. XM_001469659) was isolated from genomic DNA of *L. infantum* PCR using sense (5'-GTCGCTTCTGGACATCCTTG-3') and antisense (5'-CAT TGGCAGAGAACATCTGC-3') primers. Nucleotide sequences were determined by PCR using sense (5'-CACCCGGGATGTCTTCTCCAGCGCCAC; bears the BglII site) primers.

**Results**

**Chloroquine accumulation and efflux.** Chloroquine accumulation was determined by measuring the decrease in fluorescence intensity after the incubation with parasites. The fluorescence of 2 μM chloroquine in HPIM buffer (pH 8.5) was measured at 28°C. After stabilization of the fluorescence signal, parasites were added to a final concentration of 2 × 10^6 ml^-1, and the decline in fluorescence was measured as a function of time (excitation wavelength, 343 nm; emission wavelength, 382 nm; bandwidth, 4 nm) using a SLM-AMINCO fluorescein spectrophotometer (AMINCO-Bowman, Urbana, IL).

To determine chloroquine efflux, promastigotes (2 × 10^7 ml^-1) were incubated in HPIM buffer plus 50 μM chloroquine for 30 min at 28°C. After the incubation time, parasites were washed twice in cold PBS and resuspended in HPIM buffer (pH 8.5) at a final concentration of 2 × 10^6 ml^-1. Two hundred microliters was added to thermostatted (28°C) quartz cuvettes previously loaded with 1.8 ml of HPIM buffer (pH 8.5), and the increase in fluorescence was measured as described above. After the efflux, parasites were pelleted, and the fluorescence in the supernatant was measured.

**RESULTS**

**Molecular characterization and expression analysis of *L. infantum* ABCG6.** The present work focuses on the characterization of the ABCG-like gene from chromosome 36, named *L. infantum* ABCG6 according to the classification recently proposed [20]. *L. infantum* ABCG6 codes for a 683-amino-acid protein with a predicted molecular mass of 74.4 kDa. A sequence database search using the FASTA algorithm showed the best match with the ABCG/white subfamily of transporters. *L. infantum* ABCG6 shares 21.2% and 24.1% amino acid identity with human ABCG1 and ABCG2, respectively, 23.8% with the *Drosophila white* gene product, and 25.7% with yeast ADP1. The highly conserved NBD shares...
50% identity with those of human ABCG1 and ABCG2. Hydrophobicity plots of LiABCG6 showed the expected topology for an ABCG half-transporter with a hydrophilic region, bearing the highly conserved motifs of ABC transporters at the NH2 terminus and a hydrophobic region (TMD) with six transmembrane segments at the COOH terminus.

Localization of LiABCG6 in *Leishmania* promastigotes. To ascertain the subcellular localization of LiABCG6, GFP chimeras were created. Expression was determined by Western blotting (Fig. 1A). During the chimera selection process, high levels of expression were achieved, even at the minimum concentration of selectable drug (25 μg/ml) (data not shown).

Localization studies performed on GFP-LiABCG6- and LiABCG6-GFP-transfected promastigotes by fluorescence microscopy showed different localizations of LiABCG6, depending on the end to which GFP was fused. In parasites transfected with GFP-LiABCG6, the protein was localized mainly at the PM (Fig. 1B). This was verified with “ghost” parasites, in which the protein could still be observed at the PM. However, parasites transfected with LiABCG6-GFP showed a different localization of LiABCG6: the protein was localized exclusively at the multivesicular tubule, considered a terminal lysosome (22) (Fig. 1B).

Fluorescent phospholipid uptake in parasites overexpressing LiABCG6. To elucidate the possible role of LiABCG6 in lipid transport, we investigated the internalization of fluorescent phospholipid analogues in parasites transfected with LiABCG6-pXG2. LiABCG6 overexpression was analyzed by Northern blotting (data not shown). The accumulation of
NBD-PC, NBD-PE, and NBD-PS was significantly lower in parasites overexpressing LiABCG6 than in mock-transfected parasites (Fig. 2). No significant differences in size were observed between mock and LiABCG6 transfectants by flow cytometry analysis (n = 4) (data not shown). The ratios of accumulated NBD-lipids between mock-transfected and LiABCG6-overexpressing parasites were 1.80 ± 0.09 for NBD-PC, 1.58 ± 0.19 for NBD-PE, and 1.42 ± 0.02 for NBD-PS (n = 4; P < 0.005). No significant differences were observed for NBD-SM (ratio, 0.98 ± 0.03; n = 4). No differences in the uptake of the endocytic marker FM4-64 were observed between mock-transfected and LiABCG6-overexpressing parasites (data not shown). Taken together, these results suggest that LiABCG6 activity specifically affects the accumulation of NBD-lipids by enhanced outward transport across the PM.

Drug sensitivity profiles of *Leishmania* overexpressing LiABCG6. Since several ABC transporters have been implicated in drug resistance in *Leishmania*, we analyzed whether overexpression of LiABCG6 could confer resistance to several drugs. Different unrelated leishmanicidal drugs were tested, including alkyl-phospholipids (miltefosine, edelfosine, and perifosine), azoles (ketoconazole), aminoquinolines (sitamaquine, chloroquine, mefloquine, and primaquine), and camptothecin (Fig. 3). Parasites overexpressing LiABCG6 were about twofold more resistant to miltefosine, edelfosine, and perifosine than mock-transfected parasites, pointing to alkyl-phospholipids as possible substrates for LiABCG6 (Table 1). Overexpression of LiABCG6 conferred resistance to the aminoquinolines sitamaquine, chloroquine, and (slightly) mefloquine, whereas it did not affect susceptibility to primaquine (Table 1). These results suggest that aminoquinolines are putative LiABCG6 substrates. LiABCG6 is also involved in camptothecin resistance, as previously reported for *L. donovani* ABCG6 (5). LiABCG6 did not confer resistance to ketoconazole. Promastigotes overexpressing GFP-LiABCG6 showed behavior similar to that of parasites overexpressing untagged LiABCG6. Therefore, tagging at the NH₂ terminus did not interfere with functionality, whereas a tag at the COOH terminus inactivated the protein functionality while also altering the correct localization (Table 1 and Fig. 1B).

**Miltefosine and sitamaquine accumulation in *Leishmania* promastigotes overexpressing LiABCG6.** To corroborate that LiABCG6 would have alkyl-phospholipid analogues and aminoquinolines as potential substrates, the intracellular accumulation of [¹⁴C]miltefosine and [¹⁴C]sitamaquine was measured. Parasites overexpressing LiABCG6 accumulated 75% and 70% of the levels of miltefosine and sitamaquine accumulated by mock-transfected parasites, respectively (Fig. 4A and B). We consider that these reductions in drug accumulation are compatible with ~2-fold increased IC₅₀s, comparable to those previously described for *Leishmania* ABCG4 (6).

These results are consistent with enhanced outward transport of both [¹⁴C]miltefosine and [¹⁴C]sitamaquine across the PM, probably due to the efflux activity of LiABCG6.

**Chloroquine accumulation and efflux.** To further corroborate that chloroquine is a putative substrate for LiABCG6, a transport assay was developed. Chloroquine is a diprotic weak base, only slightly fluorescent at a pH below 7.5, but its quantum yield increases severalfold at a basic pH (Fig. 5A). We therefore hypothesized that it could be possible to measure real-time chloroquine transport as a change in the fluorescence

<table>
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<tr>
<th>Drug</th>
<th>IC₅₀ (µM) for parasites:</th>
<th>RI [c]</th>
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<tbody>
<tr>
<td></td>
<td>Mock transfected</td>
<td>Overexpressing LiABCG6</td>
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<tr>
<td>Miltefosine</td>
<td>6.0 ± 0.7</td>
<td>12.9 ± 1.5</td>
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<tr>
<td>Edelfosine</td>
<td>4.8 ± 0.6</td>
<td>11.3 ± 0.2</td>
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<tr>
<td>Perifosine</td>
<td>3.8 ± 0.6</td>
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<tr>
<td>Sitamaquine</td>
<td>7.4 ± 0.6</td>
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<tr>
<td>Chloroquine</td>
<td>6.4 ± 1.2</td>
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<tr>
<td>Mefloquine</td>
<td>5.2 ± 0.8</td>
<td>7.0 ± 0.9</td>
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<td>Primaquine</td>
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<td>Ketoconazole</td>
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<tr>
<td>Camptothecin</td>
<td>17.1 ± 1.5</td>
<td>34.4 ± 1.8</td>
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*a* Parasites were grown as described in Materials and Methods for 72 h at 28°C in the presence of increasing concentrations of drugs. Subsequently, cell viability was determined using an MTT-based assay. N/D, not determined.

*b* Data are means ± standard deviations from six independent experiments.

*c* RI, resistance index, calculated by dividing the IC₅₀ for LiABCG6-overexpressing parasites by that for mock-transfected parasites. Significant differences from results for parasites harboring the pXG control vector were determined by Student’s test (*, P < 0.001).
ments performed in duplicate; error bars, standard deviations. Signif-

percentages of 

percentage of 

the amount incorporated by the mock-transfected parasites is taken as 

normalized to the protein concentration. Data are expressed as the 

washed with cold PBS, which, in the case of [14C]miltefosine, contained 

After 30 min and 60 min, respectively, parasites were chilled on ice and 

PHMI buffer, pH 7.4, with 50 mM HCl. 

activity at the PM. 

pressing LiABCG6 was slower and less than that in mock-

increase in fluorescence intensity from LiABCG6-overexpress-

in HPMI buffer, pH 8.5. A faster increase in fluorescence intensity from LiABCG6-overexpressing parasites than from mock-transfected parasites was noticed, due to chloroquine accumulation in 

stimulating the process in a spectrofluorimeter. Drug accumulation 

of live parasites in a medium at pH 8.5 by continuously moni-

would be measured as a decrease in chloroquine fluorescence, 

because the drug would enter a more acidic compartment. Drug efflux from cells that have previously internalized a dye would be measured as an increase in fluorescence due to the exit of the drug to the more basic external medium. 

In order to prove this, we first measured chloroquine fluo-

rescence in HPMI buffer (pH 8.5). After several seconds, par-

as were added, and a time-dependent decline in fluorescence 

could be observed, due to chloroquine accumulation in promastigotes, with a pH lower than that of the external medium. Chloroquine accumulation in parasites overexpressing LiABCG6 was slower and less than that in mock-transfected parasites (Fig. 5B), probably due to LiABCG6 activity at the PM. 

Chloroquine efflux was measured by incubating the parasites in HPMI buffer, pH 7.4, with 50 μM chloroquine for 30 min at 28°C. Parasites were washed and resuspended in HPMI buffer, pH 8.5, and fluorescence was measured (Fig. 5C). A faster increase in fluorescence intensity from LiABCG6-overexpressing parasites than from mock-transfected parasites was observed, consistent with protein-mediated translocation of chloroquine to the medium, where chloroquine becomes fluorescent. To further corroborate this, parasites were pelleted after the efflux, and the fluorescence of the supernatant was measured. The fluorescence of the LiABCG6 supernatant was twice that of the mock-transfected parasite supernatant (Fig. 5C inset). We can conclude that this efflux activity correlated well with a fourfold increase in IC₅₀ for chloroquine in parasites overexpressing LiABCG6. 

DISCUSSION 

In this report we provide evidence for the lipid and drug transport activity of the ABCG-like half-transporter LiABCG6 in the protozoan parasite L. infantum. 

Parasites overexpressing LiABCG6 accumulated significantly lower levels of fluorescent phospholipid analogues of PC, PE, and PS than control cells. The reduction in phospholipid accumulation was equivalent to that observed for other ABC transporters from Leishmania (2, 24). However, no accumulation differences were noticed in parasites labeled with the sphingolipid analogue or with the endocytic/exocytic marker FM4-64. Therefore, the most straightforward explanation for these results would be enhanced outward transport of these lipid analogues across the PM by LiABCG6. 

Parasites overexpressing LiABCG6 were resistant to the alkyl-phosphocholine derivatives miltefosine and perifosine, as well as to the alkyl-glycerophospholipid edelfosine. In agreement with this, parasites overexpressing LiABCG6 showed significantly reduced levels of [14C]miltefosine accumulation. Members of the Leishmania ABCA family are implicated in phospholipid trafficking in Leishmania, though they do not confer resistance to alkyl-phospholipids (2, 24). However, the Leishmania ABCB1 transporter (LtrMDR1) and LiABCG4 reduce the accumulation of a PC fluorescent analogue and are implicated in resistance to miltefosine (6, 28, 30). 

We had previously reported that overexpression of an ABCG4 protein from Leishmania infantum confers resistance to sitamaquine (6). Parasites overexpressing LiABCG6 show a different pattern of resistance to aminoquinolines that could be attributed to the chemical structure of these drugs (Fig. 3). Chloroquine and sitamaquine have a long-chain substituent ending in a tertiary amine, whereas the substituent in primaquine ends in a primary amine. Mefloquine, which evokes slight resistance in LiABCG6-overexpressing parasites, has a substituent with a secondary amine. Furthermore, LiABCG6 confers resistance to camptothecin, as previously reported for L. donovani ABCG6 (5). Aminoquinolines and camptothecin show a quinoline ring as common motifs. Interestingly, several studies have reported that most P-glycoprotein modulators share some common chemical features, such as aromatic ring structures and a tertiary or secondary amino group (37). However, further investigation might be required. These results were further corroborated by the fact that LiABCG6-transfected parasites showed significantly reduced levels of [14C]sitamaquine accumulation. Chloroquine is a diprotic weak base that tends to accumulate in acidic compartments (16). Its fluorescence at basic pHs has allowed us to develop a novel real-time transport assay, showing that parasites overexpressing LiABCG6 accumulate less chloroquine due to increased outward transport. Finally, the ABCG4 and ABCG6 half-transporters show similar spectra of activity (reference 6 and this
work), suggesting that these transporters would act as heterodimers conferring increased resistance. Further coexpression and communoprecipitation experiments with both transporters would be needed to confirm this hypothesis.

In summary, LiABCG6 is required for the outward transport of short-chain phospholipid analogues, alkyl-phospholipids, and aminoquinolines. These findings could be of future clinical relevance, since LiABCG6, together with other ABC transporters and the miltefosine protein transport complex, contributes to resistance phenotypes in *Leishmania*.

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