Vol. 55, No. 8

Sitamaquine Overcomes ABC-Mediated Resistance to Miltefosine and Antimony in *Leishmania*[▽][†]

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Although oral miltefosine represented an important therapeutic advance in the treatment of leishmaniasis, the appearance of resistance remains a serious threat. LMDR1/LABCB4, a P-glycoprotein-like transporter included in the *Leishmania* ABC (ATP-binding cassette) family, was the first molecule shown to be involved in experimental miltefosine resistance. LMDR1 pumps drugs out of the parasite, thereby decreasing their intracellular accumulation. Sitamaquine, another promising oral drug for leishmaniasis, is currently in phase 2b clinical trials. The physicochemical features of this drug suggested to us that it could be considered for use as an LMDR1 inhibitor. Indeed, we report herein that nonleishmanicidal concentrations of sitamaquine reverse miltefosine resistance in a multidrug resistance *Leishmania tropica* line that overexpresses LMDR1. This reversal effect is due to modulation of the LMDR1-mediated efflux of miltefosine. In addition, sitamaquine is not a substrate of LMDR1, as this transporter does not affect sitamaquine accumulation or sensitivity in the parasite. Likewise, we show that ketoconazole, another oral leishmanicidal drug known to interact with ABC transporters, is also able to reverse LMDR1-mediated miltefosine resistance, although with a lower efficiency than sitamaquine. Molecular docking on a three-dimensional homology model of LMDR1 showed different preferential binding sites for each substrate-inhibitor pair, thus explaining this different behavior. Finally, we show that sitamaquine is also able to modulate the antimony resistance mediated by MRPA/LABCC3, another ABC transporter involved in experimental and clinical antimony resistance in this parasite. Taken together, these data suggest that the combination of sitamaquine with miltefosine or antimony could avoid the appearance of resistance mediated by these membrane transporters in *Leishmania*.

Drug resistance to antimonials is a major concern during the treatment of leishmaniasis, a disease caused by the protozoan parasite *Leishmania*. The therapeutic arsenal for this neglected disease was improved with the development of the first two oral drugs to treat this parasitic disease (39), namely, miltefosine, which was first marketed in India in 2002 as Impavido with the intention of replacing classical antimonial drugs as the first-line treatment in some areas of endemicity, and sitamaquine (WR6026), an 8-aminoquinoline analogue which is currently undergoing phase 2b clinical trials. However, experimental resistance to miltefosine is very easily achieved (26), and there is a serious risk of relapse after a high-dose course of miltefosine, thereby suggesting the need to introduce new therapeutic strategies, such as drug combinations, to prevent treatment failure.

Antimonial and miltefosine resistance can be conferred by the overexpression of parasite ABC (ATP-binding cassette) transporters (29). These proteins are pumps that use the energy provided by ATP hydrolysis in their nucleotide binding domains to transport different substrates that bind to their transmembrane domains. The availability of the genome sequences of *Leishmania major* and *Leishmania infantum* has highlighted the fact that *Leishmania* has 42 ABC proteins (18), some of which have been shown to be involved in experimental and/or clinical resistance (30). MRPA/PGPA (termed LABCC3 in reference 18) probably confers resistance to As^{III} and Sb^{III} by sequestering metal-thiol conjugates into intracellular vesicles (17). Additionally, MRPA has been found to be amplified in clinical antimony-resistant isolates of *Leishmania donovani* from India (25). PRP1 (LABCC7) confers resistance to pentamidine, which can be modulated by verapamil (7), whereas LABCG4 and LABCG6 confer low levels of miltefosine, sitamaquine, and camptothecin resistance (1, 3, 4). Finally, LMDR1/LABCB4, which is homologous to the human multidrug transporter P-glycoprotein (MDR1/ABCB1; Pgp), also confers a multidrug resistance (MDR) phenotype (6, 29).

LMDR1, which was the first protein found to confer miltefosine resistance in *Leishmania* (32), reduces the intracellular accumulation of miltefosine due to active drug efflux (28). LMDR1 also confers resistance to daunomycin, puromycin, vinblastine, adriamycin, and edelfosine (6, 29). Although it cannot be efficiently inhibited by classical inhibitors of human

Pgp such as verapamil and cyclosporine (29), we have previously shown that hemisynthetic flavonoids (27, 31) and β-agarofuran sesquiterpenes (33) are able to inhibit LMDR1 by binding to nucleotide binding domains and to transmembrane domains, respectively (28). These two different targets in LMDR1 have allowed the combination of low concentrations of these compounds to inhibit transporter activity (28), although both their price and difficult preparation could hamper any future clinical use. We have therefore continued our search for new agents that can reverse miltefosine resistance in *Leishmania* by considering that the ideal features of a good LMDR1 inhibitor should include, among others requirements, a low toxicity for mammalian cells, an inability to be transported by LMDR1, and, if possible, a specific leishmanicidal effect. Current drugs in clinical use or in development to treat leishmaniasis could satisfy these requirements. The oral leishmanicidal drug sitamaquine, for example, could be an interesting candidate, as different quinoline-based drugs are known to interact with some ABC transporters. Thus, Hayeshi et al. recently determined the potential inhibitory effect of many antiparasitic drugs on mammalian Pgp function (15). Interestingly, all the quinoline compounds tested (quinine, desethylamodiaquine, amodiaquine, chloroquine, and the 8-aminoquinoline primaquine) were shown to interact with Pgp, thereby suggesting that the common aromatic component of these drugs could be important in this interaction. Mefloquine and quinidine also inhibit mammalian Pgp (15, 36), and quinine and mefloquine could also be substrates of this transporter (34). Likewise, chloroquine, quinine, MK-571, primaquine, quinidine, and mefloquine interact and inhibit mammalian ABCC1/MRP1 (45, 47), with the last also inhibiting mammalian ABCC4/MRP4 (47). Additionally, *Plasmodium falciparum* Pgp (Pgh1)- and MRP-like (PfMRP) transporters are known to mediate chloroquine, mefloquine, and quinine resistance in this parasite (35, 38). Finally, LABCG4 and LABCG6 transporters confer sitamaquine resistance in *Leishmania* (3, 4).

The results reported in this work demonstrate the ability of sitamaquine and ketoconazole to overcome LMDR1-mediated miltefosine resistance in *Leishmania* without being substrates of this transporter. In addition, we show that sitamaquine is also able to circumvent MRPA-mediated antimony resistance. Thus, these compounds have the advantage of their specificity as oral leishmanicidal drugs, with low side effects at the concentrations used. Therefore, their putative future use in drug combination could represent a cost-effective way to sensitize resistant parasites and also to avoid the appearance of resistance mediated by ABC transporters.

MATERIALS AND METHODS

Chemical compounds. Daunomycin was purchased from Pfizer (Madrid, Spain), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), ketoconazole, and trivalent antimony (Sb^{III}; potassium antimony tartrate) were purchased from Sigma. Miltefosine and [^{14}C]miltefosine were obtained from Aeterna Zentaris (Frankfurt, Germany). Sitamaquine and [^{14}C]sitamaquine were purchased from GlaxoSmithKline (Greenford, United Kingdom). The 1α,2α-diacetoxy-6β,9β-difuroyloxy-4β-hydroxydihydro-β-agarofuran sesquiterpene (Macu5) was isolated from *Maytenus cuzcoina* and used as a control of the reversal agent as described previously (8).

***Leishmania* strains and cell culture.** Promastigotes from *Leishmania tropica* LRC strain (wild type [WT]) and the derivative MDR and MDR revertant

TABLE 1. Cross-resistance profile of an MDR *L. tropica* line

Drug	Line	Inhibitor	EC$_{50}$ (μM)^a	Resistance factor^b
Miltefosine	WT	—^{c}	10.6 ± 2.9	1
	MDR	—	197.3 ± 66.8	18.6*
	MDR	10 μM Macu5	9.3 ± 2.3	0.9
	MDR-Rev	—	23.8 ± 9.5	2.2*
Sitamaquine	WT	—	9.5 ± 2.1	1
	MDR	—	23.7 ± 0.3	2.5*
	MDR	10 μM Macu5	22.9 ± 1.2	2.4*
	MDR-Rev	—	21.4 ± 1.2	2.2*
Ketoconazole	WT	—	27.9 ± 7.9	1
	MDR	—	24.3 ± 3.8	0.9
	MDR	10 μM Macu5	22.0 ± 4.8	0.8
	MDR-Rev	—	31.1 ± 6.2	1.1

^{a} EC$_{50}$ values were calculated after 72 h of drug exposure at 28°C in culture medium. The results are the averages of three to seven experiments performed in triplicate ± SD.

^{b} The resistance factor is expressed as the ratio between the EC$_{50}$ of the MDR or MDR-Rev line and the EC$_{50}$ of WT line. Asterisks indicate significant differences versus controls ($P < 0.05$).

^{c} —, no inhibitor.

(MDR-Rev) lines were grown at 28°C in RPMI 1640 modified medium (Gibco) supplemented with 20% heat-inactivated fetal bovine serum (Gibco) as described previously (6, 32). The *Leishmania tarentolae* line overexpressing MRPA (*L. tarentolae* TarIIAs20,3rev+gsh1+pgpA) and its control line (*L. tarentolae* TarIIAs20,3rev+gsh1) were obtained from Marc Ouellette and were cultured as described previously (14).

Reversal effects on the resistant cell lines. The modulation of miltefosine resistance by sitamaquine and ketoconazole in the MDR *L. tropica* line was monitored as described previously (31, 32) but using 96-well plates and an MTT-based colorimetric assay. The 50% effective concentration (EC$_{50}$) is the concentration of drug that decreases the rate of cell growth by 50%; the resistance factor is expressed as the ratio of the EC$_{50}$ of the MDR line (in the presence or absence of inhibitor) to the EC$_{50}$ of the WT line. The effect of the inhibitors on the accumulation of [^{14}C]miltefosine was analyzed as described previously (28). [^{14}C]sitamaquine accumulation in WT and MDR *Leishmania* cells was determined as described previously (23). The modulation of Sb^{III} resistance by sitamaquine in *L. tarentolae* lines overexpressing MRPA (14) was monitored as described above.

Ligand docking. A homology model of LMDR1 was generated using the MODELLER 9 version 8 (37) and Amber 10 (http://ambermd.org/; D. A. Case et al., University of California, San Francisco, CA) software packages and the *Vibrio cholerae* MsbA transporter (Protein Data Bank [PDB] code: 3B5X) (46), and *Staphylococcus aureus* Sav1866 multidrug transporter (PDB code: 2HYD) (10), which share a sequence identities with LMDR1 of 24 and 28%, respectively (see Table S1 in the supplemental material), as templates. The receptor and ligands were built using Pymol version 1.2 (http://www. pymol.org), the Pymol AutoDock/Vina plug-in (41), and AutoDock Tools (40). Rigid receptor/flexible ligand docking of ketoconazole, miltefosine, and protonated sitamaquine was carried out within the LMDR1 model using the AutoDock Vina software (43). A total of 100 docked poses were generated, with an exhaustiveness parameter of 50, and the remaining parameters were set to the default settings. Subsequent flexible receptor/flexible ligand dockings were performed within the four different binding sites identified in the previous rigid receptor docking runs. A total of 20 poses were generated for each ligand in each binding site, with an exhaustiveness parameter of 50, and the remaining parameters were set to the default settings.

Statistical analysis. Statistical analyses between data were evaluated by Student's *t* test and considered significant at *P* values <0.05.

RESULTS AND DISCUSSION

Sitamaquine overcomes LMDR1-mediated miltefosine resistance by increasing intracellular miltefosine accumulation. It can be seen from Table 1 that, in agreement with published
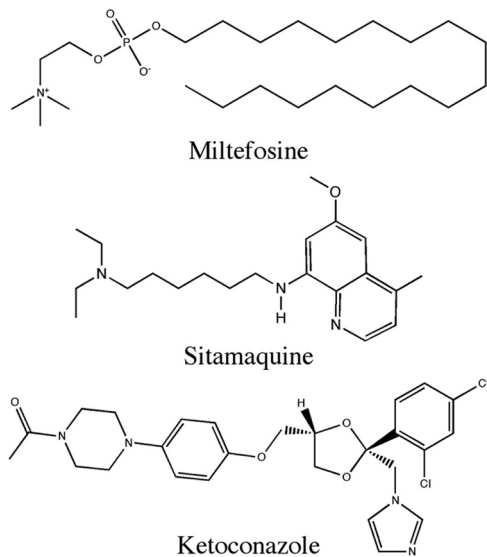
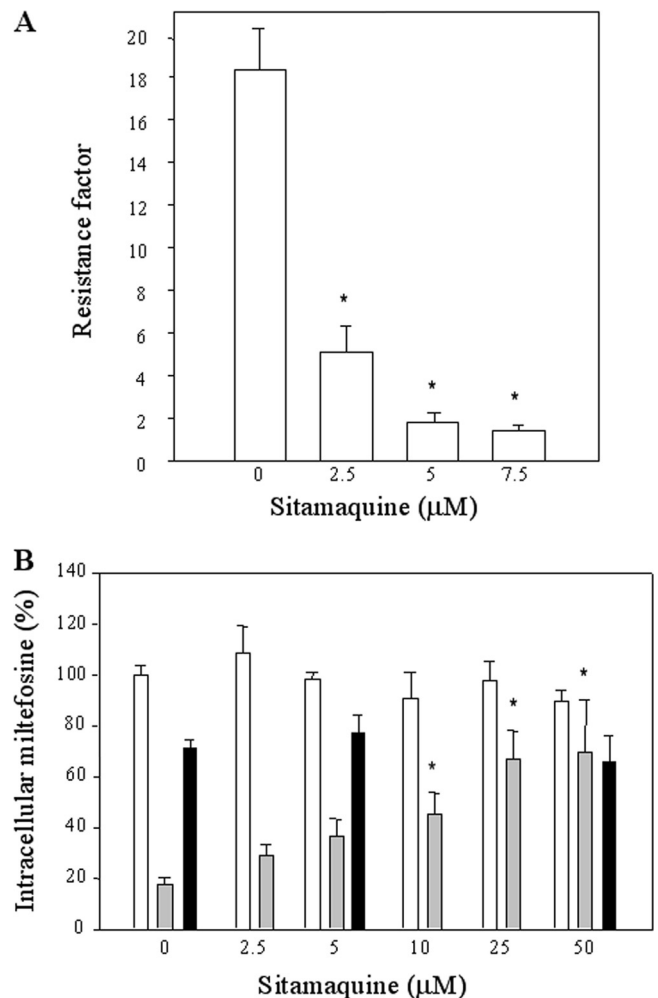FIG. 1. Chemical structures of sitamaquine, miltefosine, and ketoconazole.

FIG. 2. Modulation of LMDR1-mediated miltefosine resistance by sitamaquine in an MDR *L. tropica* line. (A) Reversion of miltefosine resistance by sitamaquine. Growth of WT and MDR parasites was determined after incubation for 72 h with increasing concentrations of miltefosine and in the presence of the indicated sitamaquine concentrations. Data representing the resistance factors (miltefosine EC_{50}s in the MDR line in the presence or absence of sitamaquine versus EC_{50}s in control WT) are means ± standard deviations (SD) of three independent experiments performed in duplicate. (B) Effect of sitamaquine on [^{14}C]miltefosine accumulation. The uptake of [^{14}C]miltefosine in WT (white bars), MDR (gray bars), and MDR-Rev (black bars) parasites was measured after 3 h in the presence of the indicated sitamaquine concentrations, as described in Materials and Methods. The data shown are the means ± SD of three independent experiments, each performed in duplicate, and are expressed as % [^{14}C]miltefosine accumulation versus the accumulation of WT controls. Asterisks indicate significant differences versus controls ($P < 0.05$).

results (32), the MDR *L. tropica* line overexpressing LMDR1 was 18.6-fold less sensitive to miltefosine (structure in Fig. 1) than the WT line. The presence of the LMDR1 inhibitor Macu5 (8) completely reverses this miltefosine-resistant phenotype (Table 1). The chemical structure of the oral leishmanicidal drug sitamaquine (Fig. 1) shows some of the characteristics previously described as being important for a human Pgp inhibitor (13), such as a conjugated planar ring, a substituted tertiary amino group, significant hydrophobicity (XLogP: 4.7 [5]), and a molecular mass of around 340 g/mol. In addition, sitamaquine is a quinoline-based compound, and, as discussed in the introduction, many drugs of this family are known to interact with mammalian Pgp and MRPs. We therefore studied the ability of sitamaquine to revert LMDR1-mediated miltefosine resistance. Figure 2A shows that sitamaquine efficiently reversed miltefosine resistance (by 75%) in a 72-h assay even at concentrations as low as 2.5 μM. Higher concentrations of sitamaquine (5 μM) completely eliminated miltefosine resistance from the MDR *Leishmania* line (Fig. 2A) without any significant cytotoxic effect (5.9% growth inhibition in the resistant line) (not shown).

We have previously shown that LMDR1-mediated miltefosine resistance in *Leishmania* is characterized by a high miltefosine efflux rate that leads to diminished drug accumulation in the parasite (28). The presence of LMDR1 inhibitors restores miltefosine accumulation in the resistant line (28). We therefore determined whether the reversal effect induced by sitamaquine was due to an increase in miltefosine accumulation in the MDR *Leishmania* line. Figure 2B shows that the accumulation values of [^{14}C]miltefosine in the MDR line were markedly increased by the presence of increasing concentrations of sitamaquine (2.5, 5, 10, 25, and 50 μM) for 3 h. These sitamaquine concentrations did not produce any effect on miltefosine accumulation in the WT and in the MDR-Rev lines (Fig. 2B) and were not toxic at the time points used (not shown). Taken together, the above results confirm that sitamaquine can be considered to be an effective reversal agent of LMDR1-mediated miltefosine resistance in *Leishmania* parasites.

Sitamaquine is not an LMDR1 substrate. One of the problems associated with most Pgp inhibitors is that they also become transported by Pgp. This means that the effective reversal concentration has to be too high, thereby producing undesirable side effects (11). In order to determine whether sitamaquine was a substrate of LMDR1, we determined the sensitiv-

ity to sitamaquine in WT and MDR *Leishmania* lines. As can be seen from Table 1, the MDR line exhibits a 2.5-fold-higher factor for resistance to sitamaquine than WT. However, the LMDR1 inhibitor Macu5 (at 10 μM) did not modify the EC$_{50}$ of the MDR line for sitamaquine, maintaining a resistance factor of 2.4. Additionally, we have determined that an MDR-Rev line that has decreased LMDR1 expression, and consequently the miltefosine- and daunomycin-resistant phenotype (32), presents EC$_{50}$s similar to those for the MDR line (Table 1). This non-LMDR1-mediated basal sitamaquine resistance level could be due to mutations generated in the adaptive selection process for this MDR line. Based on these results, we consider that sitamaquine is probably not transported by LMDR1, as further confirmed by the similar accumulations of [^{14}C]sitamaquine in both WT and MDR lines (data not shown).

LMDR1 reversal activity of ketoconazole. Ketoconazole, another oral leishmanicidal drug (structure in Fig. 1) (16) that is known to interact with different ABC transporters (44), may also be able to modulate parasite drug resistance. Furthermore, LMDR1 expression level or inhibition does not significantly modify ketoconazole sensitivity in *Leishmania* (Table 1), thereby suggesting that ketoconazole is not a substrate of LMDR1. Although ketoconazole was able to efficiently reverse miltefosine resistance in the MDR line (Fig. 3A), it was active at concentrations higher than those required for sitamaquine (Fig. 2A). Indeed, 10 μM ketoconazole produces a 75% miltefosine resistance reversal effect, similar to that produced by 2.5 μM sitamaquine. As expected, the ability of ketoconazole to revert miltefosine resistance was also due to a modulation of miltefosine accumulation in the MDR line (Fig. 3B).

Determination of different LMDR1 binding sites. Human Pgp, which was the first multidrug transporter reported (42), binds a wide spectrum of structurally different compounds from the inner leaflet of the membrane and transports them outside the cell. Multiple binding sites, where two or more molecules can bind simultaneously, within Pgp have been reported (24), thus leading to the assumption of the presence of one large binding pocket where transporter and substrates can adapt to each other in different manners (9).

In order to elucidate why sitamaquine and ketoconazole have different inhibition capabilities for LMDR1-mediated resistance, we performed molecular docking on a homology model of LMDR1. The model allows us to observe where ligands bind within the transporter and thus confirm that the equivalent human Pgp residues predicted to be accessible from the binding site (19, 21, 22) are facing toward the cavity formed by the LMDR1 model. Furthermore, in agreement with the proposed closed conformation of Pgp, residues M236 and G885 are about 20 Å apart (20). Initially we performed a fast rigid receptor/flexible ligand docking of ketoconazole, miltefosine, and sitamaquine in order to detect their putative binding sites within the LMDR1 model. These docked poses resulted in six different clusters (Fig. 4), which were grouped into four different zones (A to D) according to their locations within the model. Clusters at zones A and C are in the half part formed by transmembrane segment 4 (TM4) to TM9 and TM12, whereas clusters at zones B and D are in the other half formed by TM1 to TM3, TM6, and TM10 to TM12. Likewise, clusters at zones A and B are in the opened cavity, which is
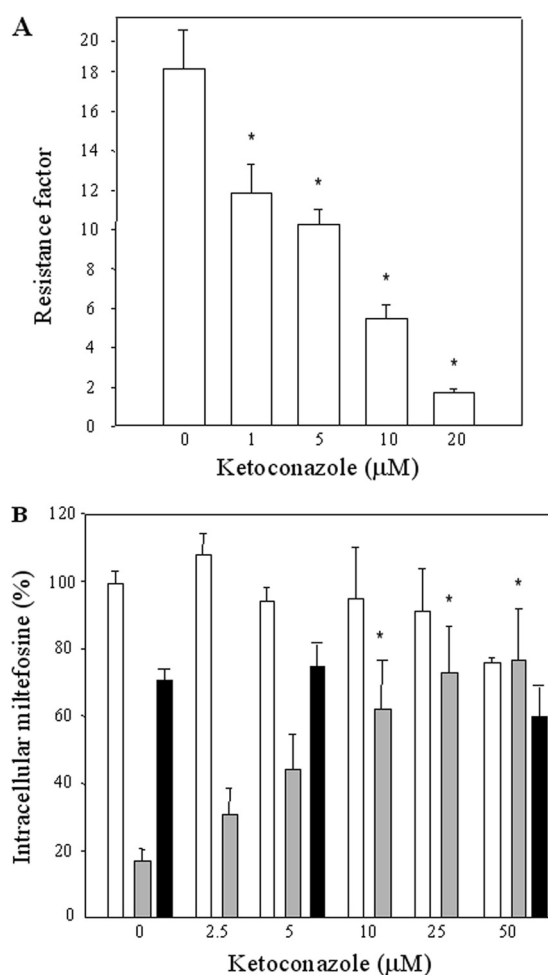

FIG. 3. Reversion of LMDR1-mediated miltefosine resistance by ketoconazole in an MDR *L. tropica* line. The reversion of miltefosine resistance by ketoconazole (A) and the effect of ketoconazole on [^{14}C]miltefosine accumulation (B) were determined as described for Fig. 2. Data are the means ± SD of three independent experiments performed in duplicate, and asterisks indicate significant differences versus the control ($P < 0.05$).

accessible from the exterior cell surface, whereas clusters at zones C and D can be reached from the inner leaflets of the membrane.

In order to determine the different receptor-ligand interactions that could explain the major miltefosine resistance reversal effect induced by sitamaquine, a more accurate flexible receptor/flexible ligand docking of the three ligands was carried out in each of zones A to D (Fig. 4). Despite the different binding energy values, the three compounds showed similar binding modes and receptor-ligand interactions in zones B to D. Interestingly, however, ketoconazole lacked the ability to interact with binding pocket A2 to the same extent as miltefosine, whereas sitamaquine mimics the poses of miltefosine (Fig. 5). Binding pocket A2 has a cavity formed by hydrophobic residues of TM5 and TM6, and their equivalent human residues are predicted to be located in the binding site (19, 21, 22) (see Table S2 in the supplemental material). Figure 5 shows how the sitamaquine (pink) and miltefosine (orange) docking

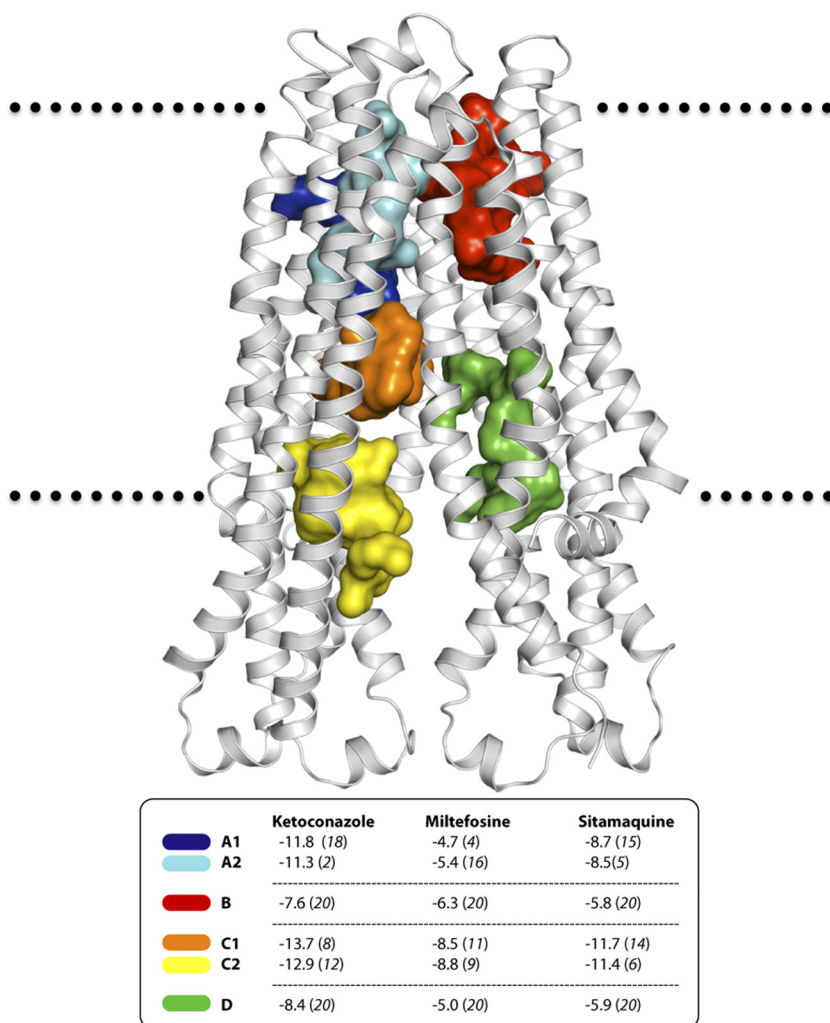|		Ketoconazole	Miltefosine	Sitamaquine
■ (blue)	A1	-11.8 (18)	-4.7 (4)	-8.7 (15)
■ (cyan)	A2	-11.3 (2)	-5.4 (16)	-8.5 (5)
■ (red)	B	-7.6 (20)	-6.3 (20)	-5.8 (20)
■ (orange)	C1	-13.7 (8)	-8.5 (11)	-11.7 (14)
■ (yellow)	C2	-12.9 (12)	-8.8 (9)	-11.4 (6)
■ (green)	D	-8.4 (20)	-5.0 (20)	-5.9 (20)

FIG. 4. Distribution of clusters within the LMDR1 model. Shown is a schematic representation of the *Leishmania* LMDR1 transmembrane domain model embedded into the cytoplasmatic membrane. Clusters A1 (blue), A2 (cyan), B (red), C1 (orange), C2 (yellow), and D (green) resulted from molecular docking. Dotted lines represent the cytoplasmic membrane boundaries. (Inset) Energy of the best ranked pose (kcal/mol) in each cluster (A to D) and, in parentheses, the number of docked poses over 20.

poses reach deeper into the hydrophobic binding cavity of A2, interacting with V367 and M364 in TM5 (21), whereas the more rigid compound ketoconazole (green) does not. Thus, these common interactions between sitamaquine and miltefosine in TM5 could explain why sitamaquine is better than ketoconazole at reversing miltefosine resistance.

Reversal activity of sitamaquine in an MRPA transporter. The observed greater effect on miltefosine resistance modulation in *Leishmania*, together with its promising leishmanicidal activity, makes sitamaquine a more interesting compound than ketoconazole in terms of future clinical use in combination therapies. As described previously, quinoline-based drugs are also able to interact with and inhibit different members of the ABC subfamily C (ABCC) transporters such as human MRP1 and MRP4 (47). We therefore determined the ability of sitamaquine to modulate the activity of MRPA, a member of the *Leishmania* ABCC subfamily involved in antimony resistance (17). As can be seen from Fig. 6A, treatment of *Leishmania* parasites overexpressing MRPA with sitamaquine at 10 or 20
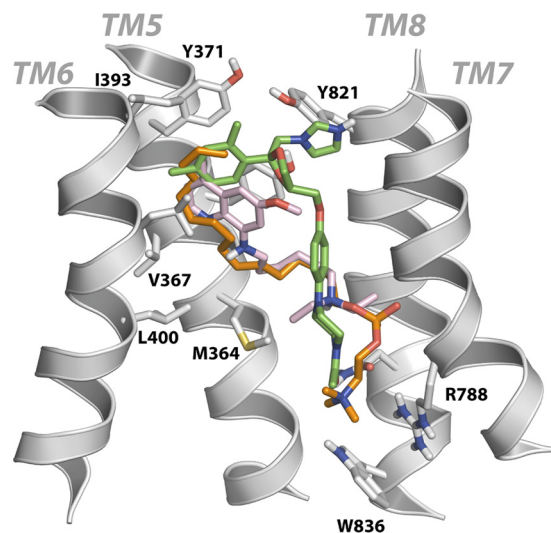

FIG. 5. Molecular docking of ligands in the LMDR1 model. Shown are docked poses of miltefosine (orange), sitamaquine (pink), and ketoconazole (green) in the A2 binding pocket of the LMDR1 model.
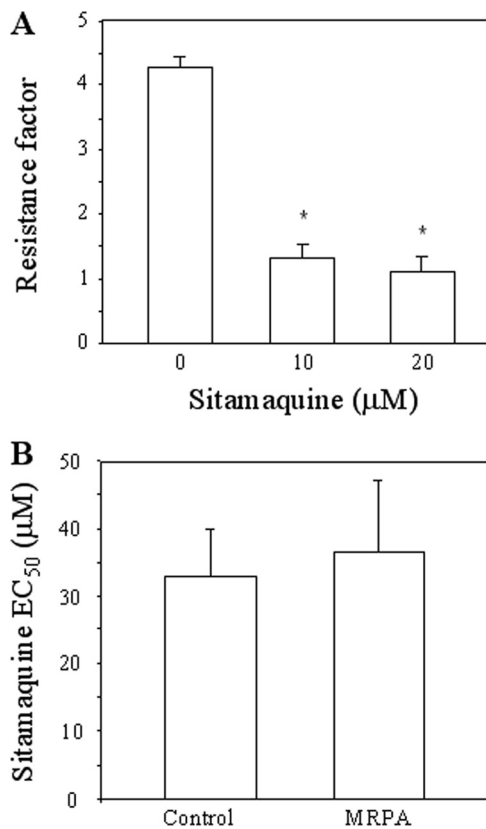
FIG. 6. Effect of sitamaquine on *Leishmania* parasites overexpressing MRPA. (A) Reversion of Sb^{III} resistance in an *L. tarentolae* line overexpressing MRPA by sitamaquine. Cell growth of control and MRPA-overexpressing parasites was determined after incubation for 72 h with different concentrations of Sb^{III} in the presence of the indicated sitamaquine concentrations. Data representing the resistance factor are means ± SD of three independent experiments performed in duplicate. Asterisks indicate significant differences versus the control ($P < 0.05$). (B) Effect of MRPA on *Leishmania* sitamaquine sensitivity. Control and MRPA-overexpressing *L. tarentolae* parasites were incubated in the presence of increasing sitamaquine concentrations for 72 h. Data representing the EC$_{50}$s are means ± SD of three independent experiments performed in duplicate.

μM lowered the 4.3-fold resistance factor for Sb^{III} to values of 1.3- and 1.1-fold, respectively. These results support the ability of sitamaquine to reverse MRPA-mediated antimony resistance in *Leishmania*. In addition, sitamaquine does not seem to be an MRPA substrate, as indirectly deduced from the similar toxicities of the parasites irrespective of their MRPA expression levels (Fig. 6B).

In conclusion, sitamaquine is an effective reversal agent for miltefosine and antimony resistance mediated by the ABC transporters LMDR1 and MRPA, respectively, in the protozoan parasite *Leishmania*. This fact, together with its efficacy as an oral leishmanicidal drug and its low side effects at the concentrations used, suggests that its future use in combination with miltefosine and/or antimony could represent a cost-effective way to avoid the appearance of ABC transporter-mediated resistance and to sensitize resistant parasites. The validation of these promising results will require the use of miltefosine- and antimony-resistant clinical *Leishmania* isolates from different areas where leishmaniasis is endemic. A similar situation has

been described for the antimalarial drug primaquine, another 8-aminoquinoline, which has a synergic activity with chloroquine against chloroquine-resistant *P. falciparum*, and the potential use of these two drugs in combination for the treatment of infections with *Plasmodium vivax* has been proposed (2, 12).

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