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Flavonoid Dimers as Bivalent Modulators for Pentamidine and Sodium Stibogluconate Resistance in \textit{Leishmania}^\textsuperscript{7}

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Drug resistance by overexpression of ATP-binding cassette (ABC) transporters is an impediment in the treatment of leishmaniasis. Flavonoids are known to reverse multidrug resistance (MDR) in \textit{Leishmania} and mammalian cancers by inhibiting ABC transporters. Here, we found that synthetic flavonoid dimers with three (compound 9c) or four (compound 9d) ethylene glycol units exhibited a significantly higher reversing activity than other shorter or longer ethylene glycol-ligated dimers, with ~3-fold sensitization of pentamidine and sodium stibogluconate (SSG) resistance in \textit{Leishmania}, respectively. This modulatory effect was dosage dependent and not observed in apigenin monomers with the linker, suggesting that the modulatory effect is due to its bivalent nature. The mechanism of reversal activity was due to increased intracellular accumulation of pentamidine and total antimony in \textit{Leishmania}. Compared to other MDR modulators such as verapamil, reserpine, quinine, quinacrine, and quinidine, compounds 9c and 9d were the only agents that can reverse SSG resistance. In terms of reversing pentamidine resistance, 9c and 9d have activities comparable to those of reserpine and quinacrine. Modulators 9c and 9d exhibited reversal activity on pentamidine resistance among \textit{LeMDR1}\textsuperscript{−/−}, \textit{LeMDR1}\textsuperscript{+/+}, and \textit{LeMDR1}-overexpressed mutants, suggesting that these modulators are specific to a non-LeMDR1 pentamidine transporter. The \textit{LeMDR1} copy number is inversely related to pentamidine resistance, suggesting that it might be involved in importing pentamidine into the mitochondria. In summary, bivalency could be a useful strategy for the development of more potent ABC transporter modulators and flavonoid dimers represent a promising reversal agent for overcoming pentamidine and SSG resistance in parasite \textit{Leishmania}.  

Leishmaniasis, one of the six major parasitic diseases targeted by the World Health Organization (WHO), is endemic in 88 countries around the world. Most leishmaniasis occurs in northern Africa, Asia, Latin America, and the Middle East. There are 350 million people at risk of infection, with 2 million cases annually. About a quarter of these cases are visceral leishmaniasis, which is the lethal form if left untreated (1). The primary treatment of leishmaniasis is by the administration of pentavalent antimonials (Pentostam and Glucantime). Secondary treatment includes pentamidine and amphotericin B. These treatments have many side effects, and their efficacies are further impeded by the emergence of clinical resistance to some of these antileishmanials (5). It has been reported that more than 50% of the visceral leishmaniasis cases in India are resistant to the antimonials (43). The WHO has stated that the resistance to pentavalent antimonials in \textit{Leishmania} is one of its top priorities (6). Newer treatments such as miltefosine, a hexadecylphosphocholine, has also shown tremendous promise. However, due to the long half-life in blood, treatment with miltefosine can easily lead to drug resistance.

ATP-binding cassette (ABC) transporters are characterized by the presence of the highly conserved ATP-binding domains. ABC transporters were first described in multidrug-resistant (MDR) cancer cells where P-glycoprotein (P-gp), a gene product of \textit{MDR1} (\textit{ABCB1}), functioned as an ATP-dependent drug efflux pump to extrude a variety of hydrophobic drugs from the cancer cells, hence reducing the intracellular drug accumulation (26). Later on, the multidrug resistance-associated protein (MRP1 encoded by \textit{ABCC1}) was found to be another ABC transporter that can also mediate the efflux of drugs and causes MDR (47). Both P-gp and MRP consist of two homologous halves, each composed of a transmembrane domain (TMD), involved in drug binding and efflux, and a cytosolic nucleotide-binding domain (NBD), with characteristic Walker A and B motifs involved in ATP binding and hydrolysis (45). Hydrolysis of ATP is tightly coupled to drug efflux. Recent evidence has shown that some P-gp (9, 11, 21, 23) and MRP (34, 35) transporters are involved in drug resistance in the protozoan parasite \textit{Leishmania} (38). Resistance to pentavalent antimonials such as sodium stibogluconate (SSG) in \textit{Leishmania tarentolae} is due to an MRP member (LtPGPA). LtPGPA may confer resistance to antimonials in promastigote cells by sequestration of the metal-thiol conjugates in an intracellular organelle located close to the flagellar pocket (30). Pentamidine is a second-line antileishmanial whose mode of action and resistance
is not well understood. It has been reported that pentamidine resistance may be due to the exclusion of pentamidine from its target, mitochondria (4). Recently, a pentamidine resistance gene (PENr), encoding a protein termed pentamidine resistance protein 1 (PRP1), has been described (12). It is also an ABC transporter and exhibited a high similarity to members of the MRP-like family (ca. 30 to 40%) (12). Resistance to miltefosine has also been suggested to be due to increased drug efflux mediated by *L. tropica* MDR1 (37).

Flavonoids constitute a group of interesting polyphenolic compounds with a wide distribution in fruits and vegetables (27, 28) and have been shown to exert a wide range of beneficial effects on human health, including protection against cardiovascular diseases and different forms of cancers (18). In the past decade, some flavonoids have been implicated in the modulation of P-gp-type MDR in cancers and shown to inhibit a variety of ATP-binding proteins such as plasma membrane ATPase (24, 44), cyclic AMP-dependent protein kinase (25), and protein kinase C (17). It is thought that the modulating activity of the flavonoids arises from competitive binding to the NBDs of P-gp through their ability to mimic the adenine moiety of ATP. On the other hand, it has been suggested that some alkyl substituted flavonoids with increased hydrophobic interactions may inhibit MDR through binding with both the steroid-interacting region and the drug binding site of TMDs in P-gp. In addition, flavonoids have also been demonstrated to inhibit daunomycin efflux and resensitize *L. tropica* to daunomycin by binding to the NBD of the P-gp-like transporter (36). Therefore, flavonoids that are consumed daily and without any detrimental side effects are attractive targets for development of novel modulators of MDR to treat both protozoan parasite *Leishmania* and cancers.

Recently, an attempt to modulate the activity of P-gp through the use of polyvalent interaction has been reported (42). Functional derivatives of stipiamide were linked via ethylene glycol chains of various lengths. It was found that polyvalency could be a useful strategy to develop more potent P-gp modulators. Using a similar strategy, we recently reported the synthesis of a series of novel bivalent flavonoid dimers based on apigenin linked by various number of ethylene glycol units (Fig. 1) (8). Apigenin was used because it is a moderate modulator of MDR in breast cancer cells (48) and has displayed a moderate affinity for the NBD2 (14). We hypothesized that a dimer will cooperatively increase the efficacy of apigenin in binding to NBD, thereby inactivating P-gp. However, without the crystal structure of the P-gp, the distance between the two NBD is unknown, even though a model has been constructed with the two NBD at a distance of about 600 nm apart (31). On the other hand, it is known that the two NBD sites move closer upon binding with ligands (41). We therefore synthesized a whole series of flavonoid dimers with various linker lengths for screening purpose. These synthetic flavonoid dimers showed a linker length-dependent inhibition of the P-gp activity in a MDR breast cancer cell line and in a resistant leukemia cell line (8). We found that compound 9d was the most potent in reversing paclitaxel resistance in a breast cancer cell line (LCC6MDR) (8).

In view of the association between P-gp expression and SSG and pentamidine resistance in *Leishmania* reported by others, we hypothesize that our synthetic apigenin dimers will also...
have similar modulating effect on the SSG and pentamidine resistance in Leishmania. In this report, we will demonstrate that the flavonoid dimers also have a length-dependent MDR-modulating activity in three Leishmania cell lines that are resistant to pentamidine and SSG.

MATERIALS AND METHODS

Cell lines and cell culture. Promastigotes of Leishmania enrietti (LePentR50, Le wild type, LeMDR1−/−, and LeMDR1-overexpressed LeV160) mutants and Leishmania donovani (LDAG83, LD2001, and LD39) were used in the present study. The former is a natural infective strain of guinea pig, and the latter is a clinical strain, which may cause visceral leishmaniasis in humans. Both strains were cultured in Schneider’s Drosophila medium (pH 6.9; Invitrogen) supplemented with 10% (vol/vol) 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).

Promastigotes of LePentR50 (pentamidine resistant, 50% inhibitory concentration [IC50] of pentamidine = 117 μg/ml), LD2001 (SSG resistant, IC50 of SSG = 4.1 mg/ml), and LD39 (SSG resistant, IC50 of SSG = 6.4 mg/ml) were cultured in the presence of 50 μg of pentamidine (Sigma)/ml and 3.5 mg of SSG (a gift from Glaxo SmithKline)/ml, respectively. No SSG was added to the L. donovani wild type (LDAG83, IC50 of SSG = 2.4 mg/ml). Promastigotes of LeV160 were cultured in the presence of 160 μg of vinblastine/ml. No pentamidine and vinblastine (Sigma) was added to the Le wild type and the LeMDR1−/− mutant.

Amastigotes of L. donovani were prepared by spinning down 50 ml of 4-day-old promastigotes (late log phase) and transferring them to an axenic medium containing M199 medium (Gibco), 0.5% Trypan casein soya, 3% l-cysteine, 15 mM d-glucose, 5 mM l-glutamine, 4 mM NaHCO3, 25 mM HEPES, 0.01 mM bathocuproine-disulfonic acid, and 0.023 mM heparin. Cells were then incubated at 37°C for 24 h. Amastigotes became osvoid in shape and were ready for drug accumulation assay.

Cell viability assay. The viability of promastigotes was determined by the Cell Titer 96 aqueous assay (Promega), which uses a novel tetrazolium compound (MTS) and electron coupling reagent, phenazine methosulfate (PMS). Promastigotes were seeded into 96-well flat bottom microtiter plate at 105 cells per well in a final volume of 100 μl of medium. For determining the cytotoxic effect of flavonoid dimers to the parasites, various concentrations of flavonoid dimers were added to the promastigotes. For determining the reversal effect of flavonoid dimers with different spacer lengths, various concentrations of antileishmanial drugs, either pentamidine or SSG, vinblastine, and puromycin was added to the wells with or without flavonoid dimers. The parasites were incubated at 27°C for 72 h. Each concentration of antileishmanials with or without synthetic modulators was tested in triplicates in each experiment. We mixed 2 mg of MTS/ml and 9.92 mg of PMS/ml in an MTS/PMS ratio of 20:1. After 72 h of incubation, 10 μl of MTS-PMS mixture was added into each well of microtiter plate. The plate was then incubated at 27°C for 4 h for color development. After 4 h of incubation, the optical density values were determined at 490 nm by using an automatic microtitre plate reader (Bio-Rad). The results were presented as a percentage of the survivors (the optical density value of each well with a test compound is divided by the value for an untreated control well).

Pentamidine accumulation assay by HPLC. The effect of flavonoid dimers on accumulation of pentamidine was investigated. Portions (1 ml) of 4-day-old promastigotes (late log phase with a cell density of about 2 × 107 cells/ml) were incubated with 0.84 mM pentamidine and various concentrations of flavonoid dimer (9d), including 0, 15, 30, and 60 μM, at 27°C for 3 h in the dark. Each concentration of 9d was tested in triplicates, and this was repeated twice in separate experiments. After 3 h of incubation, the parasites were washed three times with cold PBS (pH 7.4). The cell pellet was dissolved in 200 μl of concentrated nitric acid for 24 h at room temperature. The sample was diluted to 3 ml with distilled water, resulting in a final concentration of about 5 ppb of total Sb solution. It was then injected to inductively coupled plasma mass spectrometry (ICP-MS; Perkin-Elmer) for quantitation. Antimony was measured at its m/z ratios of 121 and 123 with indium (m/z = 115) as an internal standard. All chemicals used for the pretreatment of the samples were of at least analytical grade, and the distilled water was used directly as received without further purification (6).

RESULTS

Pentamidine-resistant L. enrietti (LePentR50) and SSG-resistant L. donovani (LD39 and LD2001). We used here three drug-resistant Leishmania cell lines, namely, LePentR50 (pentamidine-resistant L. enrietti), Ld39, and LD2001 (SSG-resistant L. donovani) to study the drug resistance-modulating activity of the synthetic flavonoid dimers. LePentR50 is a pentamidine-resistant L. enrietti cell line obtained by stepwise selection in our laboratory (unpublished data). It is maintained in the presence of 50 μg of pentamidine/ml and has an IC50 of about 117 μg/ml, whereas the wild-type L. enrietti (Le) has an IC50 of about 8.7 μg/ml (Fig. 2A). Ld39 and LD2001 are two L. donovani cell lines that are resistant to the pentavalent antimonial SSG (2). Ld39 and LD2001 are maintained in the presence of 3.5 mg SSG/ml and have an IC50 of 6.1 and 4.1 mg/ml, respectively, whereas the wild-type L. donovani (LDAG83) has an IC50 of about 2.4 mg/ml (Fig. 2B).

In vitro cytotoxicity of synthetic flavonoid dimers to Leishmania parasites. The structure of the synthetic flavonoid dimers is shown in Fig. 1. The synthesis, structural characterization, and numbering scheme of these flavonoid dimers have been reported elsewhere (8). Briefly, these flavonoid dimers are made up of two apigenin monomers linked by a biocompatible ethylene glycol linker with a different number of units (denoted by “n”). Compounds 9a to 9k-1 have n values equal to 1 to 13. We have previously suggested that each apigenin moiety of these flavonoid dimers will bind to P-gp, thereby inhibiting the pump activity (8). Compounds 10a and 10b are apigenin monomers with 3 and 4 units of ethylene glycol only.

The cytotoxicity of synthetic flavonoid dimers in each Leishmania cell line was measured by the MTS-based cell proliferation method. Table 1 summarizes the IC50 value of each synthetic modulator for LePentR50, LDAG83, and L39. Pentamidine-resistant LePentR50 was relatively resistant to synthetic flavonoid dimers (9a to 9f and 10a and 10b), with IC50 values ranging from 40 μM to greater than 200 μM. The sensitivity of L. donovani LDAG83 and Ld39 to synthetic flavonoid dimers was comparable to that of L. enrietti except for compounds 9c and 9d. It was found that both LDAG83 (IC50 of 9c = 8 ± 0.3 μM and IC50 of 9d = 7 ± 0.4 μM) and Ld39 (IC50
treated LePentR50 has an IC₅₀ of pentamidine of about 4.1 μg/ml and 9b [IC₅₀ = 39.2 ± 2.1 μg/ml; P < 0.01] significantly reduced the IC₅₀ of LePentR50 by ~3-fold (Fig. 3A). Other flavonoid dimers with either shorter linker lengths (9a [IC₅₀ = 90 ± 4.88 μg/ml and 9b [IC₅₀ = 89.2 ± 8.92 μg/ml]) or longer linker lengths (9c [IC₅₀ = 90 ± 7.88 μg/ml], 9f [IC₅₀ = 75 × 10.99 μg/ml], 9 h-1 [IC₅₀ × 100 ± 2.7 μg/ml], 9i [IC₅₀ = 73 ± 5.54 μg/ml], 9j [IC₅₀ = 134 ± 5.4 μg/ml], and 9k-1 [IC₅₀ = 130 ± 6.1 μg/ml]) gave less than half or no modulating activity (Fig. 3A). The “U”-shaped relationship between the linker length and modulating activity of the flavonoid dimers suggests that the targets of the apigenin moiety are separated by a relatively defined distance. The control compounds of apigenin monomer with three or four ethylene glycol units (10a and 10b) did not give any modulating activity even when used at double the concentration (12 μM) (Fig. 3A; IC₅₀ = 100.0 ± 5.0 μg/ml and 98.5 ± 8.5 μg/ml, respectively). This suggests that the modulating activity of compounds 9c and 9d is indeed due to their dimeric nature. A simple molar increase in the number of apigenin moiety did not result in any significant modulating activity. As a control, the linkers with n = 3 and 4 (Tri-PEG-linker and Tetra-PEG linker) did not have any reversing effect (Fig. 3A).

Effect of synthetic flavonoid dimers on modulating the SSG resistance of Ld39 and Ld2001. We have also measured the effect of synthetic flavonoid dimers on modulating SSG resistance of Ld39 and Ld2001 promastigotes. Among the synthetic flavonoid dimers (used at 6 μM), 9c and 9d were the most effective in modulating the SSG resistance of L. donovani Ld39 promastigotes. The IC₅₀ of SSG of Ld39 was reduced from 6.4 ± 0.7 mg/ml (DMSO treated) to 2.3 ± 0.2 mg/ml (9c treated) and 2.3 ± 0.3 mg/ml (9d treated) (Fig. 3B). Similar to the pentamidine resistance in LePentR50, compounds with shorter linkers (9a and 9b) or longer linkers 9e to 9k-1) did not show any significant SSG resistance modulating activity (Fig. 3B). Apigenin, 10a, and 10b, even when used at double the concentration (12 μM), also did not show any significant modulating activity (Fig. 3B). The control linkers with n = 3 (Tri-

of 9c = 11 ± 0.7 μM and IC₅₀ of 9d = 10 ± 0.9 μM) were more susceptible to 9c and 9d than was LePentR50. The species difference between L. enrietti and L. donovani (Ld39 and Ld2001). (A) LePentR50 was a pentamidine-resistant promastigote cell line selected from wild-type L. enriettii (Le) by gradually increasing the pentamidine concentration in the culture medium to 50 μg/ml. (B) Ld39 and Ld2001 were L. donovani clinical isolates known to be resistant to SSG. Wild type L. enriettii (Le) and L. donovani (LdAG83) were included for comparison. Percentage survivor was determined by MTS essay.

Effect of synthetic flavonoid dimers on modulating pentamidine resistance of LePentR50. Dimethyl sulfoxide (DMSO)-treated LePentR50 has an IC₅₀ of pentamidine of about 117.0 ± 3.0 μg/ml (Fig. 3A). A 6 μM concentration of compound 9c (n = 3; IC₅₀ = 40.0 ± 2.7 μg/ml; P < 0.01) and of 9d (n = 4; IC₅₀ = 39.2 ± 2.1 μg/ml; P < 0.01) significantly reduced the IC₅₀ of LePentR50 by ~3-fold (Fig. 3A). Other

<table>
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<th>Compound</th>
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<tr>
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</tr>
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<td>&gt;200</td>
</tr>
<tr>
<td>10b</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Apigenin</td>
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*The IC₅₀ values of each synthetic flavone were determined by an MTS-based proliferation assay. Each IC₅₀ value was derived from at least two independent experiments with triplicates in each experiment. A value of “>200” indicates that an IC₅₀ value could not be determined because these modulators did not have any cytotoxic effect at the highest concentration tested (200 μM). ND, IC₅₀ values were not determined for these modulators but no cytotoxic effect was observed at 12 μM, which was twice the concentration used to study drug resistance modulating activity.
PEG-linker) or \( n = 4 \) (Tetra-PEG-linker) did not demonstrate any effect as well (Fig. 3B).

Essentially, a similar pattern was observed when the other SSG-resistant \( L. \) donovani strain \( \text{Ld}2001 \) was studied (Fig. 3C). Compounds 9c and 9d were the most effective and can decrease the IC\(_{50}\) of SSG of \( \text{Ld}2001 \) from 6.6 mg/ml (DMSO control) to 1.5 mg/ml (9c) and 1.0 mg/ml (9d), respectively (Fig. 3C).

Interestingly, all synthetic flavonoid modulators, including 9c and 9d, had no modulatory effect on SSG-sensitive wild-type \( L. \) donovani \( \text{LdAG83} \). The IC\(_{50}\) values remained almost the same with or without any modulators (Fig. 3D). This suggests that 9c and 9d specifically target a protein that is uniquely or sufficiently present in SSG-resistant parasite but absent or rarely expressed in SSG-sensitive parasite.

Synthetic flavonoid dimers 9c and 9d show a dose-dependent modulating activity on pentamidine resistance and accumulation in \( \text{LePentR50} \). We have studied the dosage effect of the two most effective modulators, namely, 9c (containing three ethylene glycol units) and 9d (containing four ethylene glycol units) on modulating the pentamidine resistance of \( \text{LePentR50} \). When treated with only 60 \( \mu \)g of pentamidine/ml, the survival of \( \text{LePentR50} \) was only slightly decreased (94.0\% ± 2.3\% of untreated). Cotreatment of 60 \( \mu \)g of pentamidine/ml with increasing concentrations of 9c, however, resulted in a gradual
Synthetic flavonoid dimers 9c and 9d show a dose-dependent modulating activity on SSG resistance and accumulation in Ld39 cells. Similar to LePentR50, both 9c and 9d showed a dose-dependent modulating effect on the SSG resistance of Ld39 promastigotes (Fig. 6A and B). A 4 μM concentration of 9c or 9d can reduce the SSG resistance level of Ld39 back to the level of the sensitive strain of LdAG83 (Fig. 6A and B). The modulating effect of 9d was specific to a target protein present only on Ld39 because 9d did not have any modulating effect on the SSG sensitivity of LdAG83 even when used up to 6 μM (Fig. 6C).

We investigated the effect of 9d on the SSG accumulation of L. donovani amastigotes. Axenic amastigotes were produced by adapting the parasites to 37°C for 24 h. Light microscopy showed that the cells have rounded up (data not shown). We assumed that the parasites changed into the amastigote form. Other researchers have demonstrated that this adaptation method resulted in biochemical changes that were associated with the amastigote formation (39).

In the SSG accumulation experiment, we used higher concentrations of 9d (30 and 60 μM), together with a shorter incubation time (3 h) to measure the SSG accumulation. In the absence of 9d, the accumulations of SSG of Ld39 and Ld2001 were 28 and 15% of that of LdAG83, respectively (Fig. 6D). When treated with 30 μM 9d, the SSG accumulations of Ld39 and Ld2001 were increased to 74 and 83% of that of LdAG83, respectively (Fig. 6D). When the concentration of 9d was further increased to 60 μM, the SSG accumulations of Ld39 and Ld2001 were 90 and 69% of that of LdAG83, respectively (Fig. 6D). In contrast, the accumulation of SSG in SSG-sensitive LdAG83 treated with 9d (30 or 60 μM) did not significantly differ from its accumulation in cells without any treatment, indicating that the dimer 9d specifically inhibited the function of the ABC transporters present only in an SSG-resistant strain (Fig. 6D). Compound 9d did not have any cytotoxicity to L. donovani at 60 μM when treated for 3 h (data not shown), confirming that the increase in SSG accumulation was due to the modulating effect of 9d and not to its cytotoxic effect.

Comparison of the modulating activities of 9c and 9d with other traditional MDR modulators. We compared the modulating activities of 9c and 9d with verapamil, reserpine, quinine,
quinacrine, and quinidine. For LePentR50, the modulating activities of modulators of 9c (IC$_{50}$/H11005 47/H11006 1.2/H9262 g/ml) and 9d (IC$_{50}$/H11005 35/H11006 2.3/H9262 g/ml) were similar to those of reserpine (IC$_{50}$/H11005 40/H11006 1.3/H9262 g/ml) and quinacrine (IC$_{50}$/H11005 28.7/H11006 1.3/H9262 g/ml), with about 2.7-, 3.7-, 3.2-, and 4.5-fold pentamidine sensitizations, respectively (Fig. 7A). In contrast, only less than a half-fold sensitization was demonstrated when verapamil, quinine, and quinidine were used (Fig. 7A). Regarding the modulating activity of SSG resistance in Ld39, only 9c and 9d were effective (IC$_{50}$/H11005 2.3/H11006 0.1 mg/ml and 1.8/H11006 0.05 mg/ml, respectively), representing 3.1- and 3.9-fold SSG sensitization (Fig. 7B). None of the other traditional MDR chemosensitizers exhibited any modulating effect (IC$_{50}$/H11005 7.2/H11006 0.54, 7.2/H11006 0.3, 7.0/H11006 0.21, 6.7/H11006 0.11, and 7.2/H11006 0.04 mg/ml for verapamil, reserpine, quinine, quinacrine, and quinidine, respectively) (Fig. 7B).

The target of the synthetic flavonoid dimers is not LeMDR1. We are interested in identifying the target of the synthetic flavonoid dimers. Other researchers have suggested that flavonoid monomers can bind to the NBD of ABC transporter of _L. tropica_ (36). It is possible that our synthetic flavonoid dimers will also bind to the ABC transporters via the two NBDs. We have investigated whether the ABC transporter, LeMDR1, in _L. enriettii_ is the target of the synthetic flavonoid dimers. We have previously demonstrated that LeMDR1 is an ABC transporter that can mediate resistance to vinblastine and puromycin and sensitivity to rhodamine 123 (11, 16). Here we studied the modulating effect of the synthetic flavonoid dimers on three _L. enriettii_ cell lines, namely, wild-type Le, _LeMDR1_ knockout (_LeMDR1_−/−), and _LeMDR1_ overexpressed (LeV160). We found that pentamidine resistance was inversely related to the copy number of _LeMDR1_. The pentamidine IC$_{50}$/s for _LeMDR1_−/−, _Le_ wild type, and LeV160 are 18.9/H11006 0.8, 12.0/H11006 0.8, and 9.0/H11006 0.1 g/ml, respectively (Table 2). When the panel of synthetic flavonoid dimers was tested for their modulating activity on the pentamidine resistance of _LeMDR1_−/−, we found that 9c and 9d were effective in reducing the IC$_{50}$/s of pentamidine to 5/H11006 0.3 μg/ml and 4.6/H11006 0.4 μg/ml, respectively, representing 3.8- and 4.1-fold sensitizations (Table 2). Compounds 9b (IC$_{50}$/ = 9.4/H11006 0.4 μg/ml) and 9 h-1 (IC$_{50}$/ = 8.2/H11006 0.5 μg/ml) showed 2.0- and 2.3-fold sensitizations, respectively. However, 9a (IC$_{50}$/ = 18 ± 1.0 μg/ml), 9c (IC$_{50}$/ = 28.7 ± 1.3 μg/ml), and 9d (IC$_{50}$/ = 35 ± 2.3 μg/ml) were similar to those of reserpine (IC$_{50}$/ = 40 ± 1.3 μg/ml) and quinacrine (IC$_{50}$/ = 28.7 ± 1.3 μg/ml), with about 2.7-, 3.7-, 3.2-, and 4.5-fold pentamidine sensitizations, respectively (Fig. 7A). In contrast, only less than a half-fold sensitization was demonstrated when verapamil, quinine, and quinidine were used (Fig. 7A). Regarding the modulating activity of SSG resistance in Ld39, only 9c and 9d were effective (IC$_{50}$/s = 2.3 ± 0.1 mg/ml and 1.8 ± 0.05 mg/ml, respectively), representing 3.1- and 3.9-fold SSG sensitization (Fig. 7B). None of the other traditional MDR chemosensitizers exhibited any modulating effect (IC$_{50}$/s = 7.2 ± 0.54, 7.2 ± 0.3, 7.0 ± 0.21, 6.7 ± 0.11, and 7.2 ± 0.04 mg/ml for verapamil, reserpine, quinine, quinacrine, and quinidine, respectively) (Fig. 7B).

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were used at the concentration of 6×10^4 cells/well in 100 μl of LeH9262 (A) and SSG resistance of Ld39 (B). Promastigotes were seeded at 10^5 cells/well and incubated at 27°C for 72 h in the presence of either 9c, 9d, or other traditional MDR modulators. All modulators were used at the concentration of 6 μM. The IC₅₀ values were determined by using MTS assay. Each sample was tested in triplicate, and analyses were repeated three times in separate experiments.

In Le wild-type cells, 9d (IC₅₀ = 4 ± 0.3 μg/ml) significantly reduced the IC₅₀ of pentamidine from 12.0 ± 0.8 μg/ml to 4.0 ± 0.8 μg/ml (~3-fold decrease) (Table 2). In LeMDR1-overexpressed LeV160, 9c (IC₅₀ = 5.0 ± 0.4 μg/ml) and 9d (IC₅₀ = 4.7 ± 0.1 μg/ml) slightly decreased the IC₅₀ of pentamidine from 9.0 ± 0.1 μg/ml to 5.0 ± 0.4 and 4.7 ± 0.1 μg/ml, respectively (approximately 1.8-fold and 1.9-fold decreases) (Table 2). Compounds 9e (IC₅₀ = 7.5 ± 0.3 μg/ml), 9f (IC₅₀ = 7.2 ± 0.3 μg/ml), and 9i (IC₅₀ = 6.8 ± 0.2 μg/ml), however, had no sensitization effect.

The observation that the synthetic flavonoid dimers can modulate the pentamidine resistance irrespective of the copy number of LeMDR1 suggests that LeMDR1 is not the target for the synthetic flavonoid dimers. LeMDR1 is known to be responsible for vinblastine and puromycin resistance in L. enrietti (11, 16). When we tried to study the modulating activity of the flavonoid dimers on the vinblastine and puromycin resistance of LeV160, we found that none of the flavonoid dimers have any significant modulating activity (Table 2). This result further confirms that our synthetic flavonoid dimers cannot target LeMDR1.

**DISCUSSION**

Various ABC transporters in *Leishmania* have been implicated in mediating drug resistance (38). These include Lamdr1 in *L. donovani* (23), Lamdr1 and Lamdr2 in *L. amazonensis* (21, 29), LtpgpA in *L. tarentolae* (20, 22, 33), Lmdr1 in *L. tropica* (19), Lmdr1 in *L. enrietti* (11), LmepgpA in *L. mexicana* (13), LmpgpA in *L. major* (7), and PENr in *L. major* (12). Structurally, they can be grouped into the ABCB (Lamdr1, Lamdr2, Ltrmdr1, Lmdr1, and PENr) and ABCC (LtpgpA, LmepgpA, and LmpgpA) types. Both ABCB and ABCC transporters have two NBDs and therefore are potential targets of flavonoids. Indeed, flavonoids have been demonstrated to modulate the dexamethasone resistance in *L. tropica* by binding to the NBDs of LtrMDR1 (36).

Success in overcoming MDR has been limited by a lack of specificity and a low affinity of MDR modulators for the drug binding sites of ABC transporter. An application of polyvalency in drug design has recently been studied which exploits the cooperativity effect during molecular recognition and binding, resulting in a polyvalent ligand binding more tightly than equivalent monovalent system when the target protein has specific binding sites for the synthetic flavonoid dimers. LeMDR1 is known to be the major drug resistance determinant in *L. donovani* (13, 14). This suggests that in *L. tropica,* there may be an equivalent monovalent system when the target protein has specific drug-binding sites in the homo- or -heterodimeric ABC transmembrane domains (36). By binding to the NBDs of Lmdr1 (36).

**TABLE 2. Effect of synthetic flavonoid dimers on pentamidine resistance of LeMDR1 mutants**

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ of pentamidine (μg/ml) ± SD</th>
<th>Mean IC₅₀ (μg/ml) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>LeMDR1⁻⁻⁻</td>
<td>Le wild type</td>
<td>LeV160</td>
</tr>
<tr>
<td>No modulator</td>
<td>18.9 ± 0.8</td>
<td>12.0 ± 0.8</td>
</tr>
<tr>
<td>9a</td>
<td>18.0 ± 1.0</td>
<td>ND</td>
</tr>
<tr>
<td>9b</td>
<td>9.4 ± 0.4</td>
<td>ND</td>
</tr>
<tr>
<td>9c</td>
<td>5.0 ± 0.3</td>
<td>ND</td>
</tr>
<tr>
<td>9d</td>
<td>4.6 ± 0.4</td>
<td>4.0 ± 0.3</td>
</tr>
<tr>
<td>9e</td>
<td>12.5 ± 0.1</td>
<td>ND</td>
</tr>
<tr>
<td>9f</td>
<td>12.5 ± 0.8</td>
<td>ND</td>
</tr>
<tr>
<td>9h-1</td>
<td>8.2 ± 0.5</td>
<td>ND</td>
</tr>
<tr>
<td>9l</td>
<td>13.8 ± 0.7</td>
<td>ND</td>
</tr>
<tr>
<td>9j</td>
<td>20.9 ± 1.3</td>
<td>ND</td>
</tr>
<tr>
<td>9k-1</td>
<td>20.9 ± 3.0</td>
<td>ND</td>
</tr>
</tbody>
</table>

* The IC₅₀ values for each drug were determined by a MTS-based proliferation assay. Each IC₅₀ value was derived from at least three independent experiments with triplicates in each experiment. ND, not determined.
to enhance the efficacy of MDR modulators. In the present study, we used dimers of flavonoids that differ only in the length of ethylene glycol (from one ethylene glycol unit to thirteen ethylene glycol units) to investigate whether polyvalency is a practical strategy to develop inhibitors for the ABC transporter-mediated pentamidine and SSG resistance in the parasite Leishmania.

Pentamidine resistance in Leishmania may be caused by the exclusion of pentamidine from mitochondria in L. mexicana (3) and in L. donovani (32). A genetic approach has identified an ABC transporter PRP1 that may be involved in pentamidine resistance (12). It is possible that multiple factors are involved in pentamidine resistance. Here we have used a stepwise selected pentamidine-resistant L. enrietti cell line (LePentR50) to investigate the molecular mechanism of pentamidine resistance. First, the pentamidine resistance factor may be an ABC transporter because our synthetic flavonoid dimer, particularly 9c and 9d, can modulate the pentamidine resistance of LePentR50 in a dose-dependent manner. Flavonoids have been demonstrated to be an efficient MDR modulator in both mammalian P-gp (15) and Leishmania ABC transporters (36) by binding to the NBDs. We assume that our synthetic apigenin dimers may also bind to the NBDs, although we do not have any direct proof yet. We have previously demonstrated that our synthetic flavonoid dimers can inhibit P-gp-mediated anticancer drug resistance in mammalian cells by increasing drug accumulation (8). Such observations support our hypothesis that our synthetic flavonoid dimers might indeed bind to a putative ABC transporter in Leishmania. Second, this putative ABC transporter is not LeMDR1 because our flavonoid dimers can modulate the pentamidine resistance irrespective of the LeMDR1 copy number (LeMDR1−/−, Le, or LeV160). This is further confirmed by the observation that the LeMDR1-mediated vinblastine and puromycin resistance are not affected by the flavonoid dimers. The identity of this ABC transporter remains elusive. In addition to this ABC transporter, there may be other factors that might contribute to pentamidine resistance in L. enrietti. LeMDR1 could be one of the factors. Here we found that pentamidine resistance is inversely associated with LeMDR1 copy number. The IC_{50} of pentamidine of LeMDR1−/−, Le, and LeV160 are 18.9 ± 0.8, 12.0 ± 0.8, and 9.0 ± 0.1 μg/ml, respectively (Table 2). We have previously reported that LeMDR1 is inversely associated with rhodamine resistance, and LeMDR1 may be sequestering to a multivesicular tubules that could connect to mitochondria (16). Therefore, LeMDR1 overexpression results in concentrating rhodamine 123 with its mitochondrial target and causes hypersensitivity. Based on our observation here, we hypothesize that LeMDR1 may be working similarly on causing pentamidine hypersensitivity by concentrating pentamidine with its mitochondrial target(s). In such a case, LeMDR1 may be the factor involved in the accumulation of pentamidine indirectly into mitochondria. In summary, we hypothesize that the pentamidine resistance in L. enrietti may be caused by an ABC transporter involved in lowering pentamidine accumulation. In addition, LeMDR1 is involved in importing pentamidine indirectly into mitochondria, possibly via multivesicular tubules.

Regardless of the identity of the putative ABC transporter that causes pentamidine resistance in LePentR50, our synthetic flavonoid dimers can inhibit it and reverse the pentamidine resistance. Compounds 9c or 9d with two apigenins connected by three or four ethylene glycol units exhibited the highest modulating activity of both pentamidine and SSG resistance, with an ~3-fold decrease in IC_{50}. Other flavonoid dimers with longer or shorter linker lengths showed a lower activity or no modulating activity. The apigenin monomers with the same number of ethylene glycols in the linker (10a and 10b) did not have any modulating activity, even when twice the concentration was used (12 μM). This clearly demonstrates that the modulatory activity of 9c and 9d is not due to the doubled concentration of the flavonoid binding to the ABC transporters but rather due to the chain length effect of the ethylene glycol units between the two apigenins. The optimal chain length is three to four ethylene glycol units. This result suggests that the two apigenin targets of the transporter have a relatively optimal distance between them. Only when the flavonoid dimers have the suitable length (three to four ethylene glycol units) will they be able to bind to them tightly. Previously, we have reported that the optimal linker length to modulate paclitaxel resistance in human breast cancer cells was also three to four ethylene glycols, suggesting the transporter in L. enrietti and L. donovani involved in pentamidine and SSG resistance is likely to be an ABC transporter and may have a similar structure as the human P-gp (8). The distance between the two apigenin targets will have a similar distance between them.

9c and 9d work to reverse the pentamidine and SSG resistance by increasing drug accumulation in the resistant cells. Treatment with 9c and 9d resulted in a dose-dependent increase in the accumulation of pentamidine and SSG. This result also indirectly suggests that an efflux transporter is mediating pentamidine and SSG resistance by lowering the drug accumulation. We are assuming that such an efflux transporter is an ABC transporter and that it is the target of 9c and 9d. At this point, we do not know where the flavonoid dimers are binding to the putative ABC transporter. Flavonoids have been demonstrated to bind to a region that is overlapped by the ATP-binding and the steroid-binding region. However, we have no experimental evidence to show that the flavonoid dimer is binding to the same site at which the monomer binds. The target could either be the NBD or the drug binding site. In the former case, the flavonoid dimer will inhibit the ATPase activity, whereas in the latter case the flavonoid dimer will act as a competitive inhibitor.

In comparison with other traditional MDR modulators, 9c and 9d exhibited a pentamidine resistance reversal activity comparable to that of reserpine and quinacrine. In the case of SSG resistance, only 9c and 9d have significant modulating activity, whereas none of the traditional MDR modulators work. This demonstrates that polyvalency is indeed a powerful approach in designing novel MDR modulators. An application of polyvalency in drug design has recently been studied that exploits the cooperativity effect in molecular recognition and binding (10, 40, 42, 46). Our study now demonstrates that the bivalent nature of flavonoid synthesized in the present study can dramatically increase the reversal activity of modulators, so it is of great significance for future clinical application.

In summary, our study demonstrates that dimerization of flavonoids using spacers of a defined ethylene glycol units can enhance the reversal activity of modulators on antileishmanial