Flavonoid Dimers as Bivalent Modulators for Pentamidine and Sodium Stibogluconate Resistance in *Leishmania*  

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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 *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 *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 *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 *LeMDR1*/+, *LeMDR1*/+, and *LeMDR1*-overexpressed mutants, suggesting that these modulators are specific to a non-LeMDR1 pentamidine transporter. The *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 *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 *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 *MDR1* (*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 *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 *Leishmania* (38). Resistance to pentavalent antimonials such as sodium stibogluconate (SSG) in *Leishmania tarentolae* is due to an MRP member (*LiPgpA*). *LiPgpA* 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* cells that are resistant to pentamidine and SSG.

**MATERIALS AND METHODS**

**Cell lines and cell culture.** Promastigotes of *Leishmania enrietti* (LePentR50, Le wild type, LeMDR<sup>1</sup>/H<sub>11002</sub> and LeMDR<sup>1</sup>/overexpressed LeV160) 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.

**Mammalian cell lines.** The promastigotes of *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 wild type (LdAG83, IC<sub>50</sub> of SSG<sub>H11005</sub> 4.1 mg/ml), and Ld39 (SSG resistant, IC<sub>50</sub> of SSG<sub>H11005</sub> 11.7 mg/ml) was used as a control cell line obtained by stepwise selection in our laboratory (unpublished data). It is maintained in the presence of 3.5 mg SSG/ml and has an IC<sub>50</sub> of about 117 μg/ml, whereas the wild-type *L. enrietti* (Le) has an IC<sub>50</sub> of about 8.7 μg/ml (Fig. 2A). Ld39 and Ld2001 are two *L. donovani* cell lines that are resistant to the pentavalent antimonal SSG (2). Ld39 and Ld2001 are maintained in the presence of 3.5 mg SSG/ml and have IC<sub>50</sub> of 6.1 and 4.1 mg/ml, respectively, whereas the wild-type *L. donovani* (LdAG83) has an IC<sub>50</sub> of about 2.4 mg/ml (Fig. 2B).

**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 IC<sub>50</sub> of about 117 μg/ml, whereas the wild-type *L. enrietti* (Le) has an IC<sub>50</sub> of about 8.7 μg/ml (Fig. 2A).

**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 IC<sub>50</sub> value of each synthetic modulator for LePentR50, LdAG83, and Ld39. Pentamidine-resistant LePentR50 was relatively resistant to synthetic flavonoid dimers (9a to 9f and 10a and 10b), with IC<sub>50</sub> 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 9e and 9d. It was found that both LdAG83 (IC<sub>50</sub> of 9e = 8 ± 0.3 μM and IC<sub>50</sub> of 9d = 7 ± 0.4 μM) and Ld39 (IC<sub>50</sub> not be eluted out under these conditions. To generate a standard curve, a 200 μM stock solution of pentamidine isethionate salt were prepared by dissolving 2.5 mg of pentamidine isethionate salt in 21 ml of 75% ACN (10 mM TMAC, 10 mM SHS, 4.2 mM PA). Concentrations of 100, 50, 25, and 13 μM were then incubated with 0.05 mM SSG and various concentrations of flavonoid dimer (9d), including 0, 30, and 60 μM, at 37°C for 3 h. Each concentration of 9d was tested in triplicates in different 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 subjected 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 (In, 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).

**Total antimony (Sb(III) and Sb(V)) accumulation assay using ICP-MS.** The effect of flavonoid dimers on accumulation of antimony SSG was investigated. Amastigotes are more susceptible to SSG and therefore accumulate more SSG compared to promastigotes. We therefore chose to use amastigotes to study the Sb accumulation assay. A 1-ml portion of 4-day-old amastigotes (2 × 10<sup>5</sup> cells/ml) was incubated with 0.05 mM SSG and various concentrations of flavonoid dimer (9d), including 0, 30, and 60 μM, at 37°C for 3 h. 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 subjected 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 (In, 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).
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], 9h-1 [IC50 × 106 ± 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. enriettii and L. donovani was limited to the apigenin dimers 9c and 9d only. These two species were equally sensitive to apigenin monomer and apigenin with three (10a) or four (10b) ethylene glycol units (Table 1). The hypersensitivity of L. donovani, both LdAG83 and Ld39, to compounds 9c and 9d may mean that these two apigenin dimers may be useful as an anti-L. donovani agent. Indeed, we have previously demonstrated that 5 μM concentrations of 9c and 9d were nontoxic to mammalian cancer cells in vitro (8). In the studies described below, we used 6 μM concentrations of synthetic flavonoid dimers to test their modulating effect on the drug resistance in LePentR50, Ld39, and Ld2001.

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

![FIG. 2. Drug-resistant Leishmania used in the present study: pentamidine-resistant L. enriettii (LePentR50) and SSG-resistant 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.](Downloaded from http://aac.asm.org/ on June 26, 2017 by guest)

**TABLE 1. IC50 of synthetic flavonoids for Leishmania parasites**

<table>
<thead>
<tr>
<th>Compound</th>
<th>LePentR50</th>
<th>LdAG83</th>
<th>Ld39</th>
</tr>
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<tbody>
<tr>
<td>9a</td>
<td>&gt;200</td>
<td>95 ± 3.2</td>
<td>117 ± 10</td>
</tr>
<tr>
<td>9b</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>9c</td>
<td>&gt;200</td>
<td>8 ± 0.3</td>
<td>11 ± 0.7</td>
</tr>
<tr>
<td>9d</td>
<td>&gt;200</td>
<td>7 ± 0.4</td>
<td>10 ± 0.9</td>
</tr>
<tr>
<td>9e</td>
<td>70 ± 3.0</td>
<td>30 ± 1.2</td>
<td>42 ± 2.3</td>
</tr>
<tr>
<td>9f</td>
<td>40 ± 5.3</td>
<td>11 ± 2.0</td>
<td>13 ± 0.6</td>
</tr>
<tr>
<td>9h-1</td>
<td>ND</td>
<td>12 ± 0.2</td>
<td>14 ± 0.1</td>
</tr>
<tr>
<td>9i</td>
<td>ND</td>
<td>10 ± 0.3</td>
<td>14 ± 0.1</td>
</tr>
<tr>
<td>9j</td>
<td>ND</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>9k-1</td>
<td>ND</td>
<td>50 ± 7</td>
<td>60 ± 3</td>
</tr>
<tr>
<td>10a</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>10b</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Apigenin</td>
<td>55 ± 2.6</td>
<td>32 ± 4.1</td>
<td>43 ± 5.9</td>
</tr>
</tbody>
</table>

*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 \( \text{Ld2001} \) was studied (Fig. 3C). Compounds \( 9c \) and \( 9d \) were the most effective and can decrease the \( IC_{50} \) of SSG of \( \text{Ld2001} \) 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\% \pm 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,
We are interested in identifying the target of the synthetic flavonoid dimers. We found that pentamidine resistance was inversely related to the copy number of LeMDR1, and sensitivity to rhodamine 123 (11, 16). Here we studied the modulating effect of the synthetic flavonoid dimers on the ABC transporter, LeMDR1, in L. enriettii wild type, and LeV160, which were overexpressed (LeMDR1/−/−). 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} for LeMDR1/−/−, Le wild type, and LeV160 are 18.9 ± 0.8, 12.0 ± 0.8, and 9.0 ± 0.1 μg/ml, respectively (Table 2). When the panel of synthetic flavonoid dimers was tested for their modulating effect on the pentamidine resistance of LeMDR1/−/−, we found that 9c and 9d were effective in reducing the IC_{50} of pentamidine to 5 ± 0.3 μg/ml and 4.6 ± 0.4 μg/ml, respectively, representing 3.8- and 4.1-fold sensitizations (Table 2). Compounds 9b (IC_{50} = 9.4 ± 0.4 μg/ml) and 9 h-1 (IC_{50} = 8.2 ± 0.5 μg/ml) showed 2.0- and 2.3-fold sensitizations, respectively. However, 9a (IC_{50} = 18 ± 1.0 μg/ml), 9e (IC_{50} = 28.7 ± 1.3 μg/ml) were more effective. For LePentR50, the modulating activities of modulators of 9c (IC_{50} = 47 ± 1.2 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} = 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} = 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).

The target of the synthetic flavonoid dimers is not LeMDR1. We have investigated whether the ABC transporter, LeMDR1, in L. tropica (36). It is possible that our synthetic flavonoid dimers will also bind to the ABC transporters via the two NBDs. 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} for LeMDR1/−/−, Le wild type, and LeV160 are 18.9 ± 0.8, 12.0 ± 0.8, and 9.0 ± 0.1 μg/ml, respectively (Table 2). When the panel of synthetic flavonoid dimers was tested for their modulating effect on the pentamidine resistance of LeMDR1/−/−, we found that 9c and 9d were effective in reducing the IC_{50} of pentamidine to 5 ± 0.3 μg/ml and 4.6 ± 0.4 μg/ml, respectively, representing 3.8- and 4.1-fold sensitizations (Table 2). Compounds 9b (IC_{50} = 9.4 ± 0.4 μg/ml) and 9 h-1 (IC_{50} = 8.2 ± 0.5 μg/ml) showed 2.0- and 2.3-fold sensitizations, respectively. However, 9a (IC_{50} = 18 ± 1.0 μg/ml), 9e (IC_{50} = 28.7 ± 1.3 μg/ml) were more effective. For LePentR50, the modulating activities of modulators of 9c (IC_{50} = 47 ± 1.2 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} = 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} = 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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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.

### DISCUSSION

Various ABC transporters in *Leishmania* have been implicated in mediating drug resistance (38). These include *Lmdr1* in *L. donovani* (23), *Lamdr1* and *Lamdr2* in *L. amazonensis* (21, 29), *LtpgpA* in *L. tarentolae* (20, 22, 33), *Lmdr1* in *L. tropica* (19), *Lemdr1* in *L. enriettii* (11), *LmepgpA* in *L. mexicana* (13), *LmpgpA* in *L. major* (7), and PEN* in *L. major* (12). Structurally, they can be grouped into the ABCB, ABCG, and ABCC transporters. *L. tropica* and *L. mexicana* are the most extensively studied in terms of their drug resistance mechanisms. These studies have identified several ABC transporters that are involved in drug resistance. For example, *Lmdr1* and *Lamdr1* are involved in the resistance of *L. major* to various drugs, including pentamidine. The presence of these transporters in *L. tropica* and *L. mexicana* suggests that similar mechanisms may be involved in drug resistance in these species.

The results of our study indicate that the synthetic flavonoid dimers can modulate the pentamidine resistance of *Leishmania* strains in a manner similar to that observed with other MDR transporters. The flavonoid dimers were found to be effective in modulating the IC₅₀ values of pentamidine in *Leishmania* strains, and the IC₅₀ values were determined by using MTS assay. Each sample was tested in triplicate, and the IC₅₀ values were derived from at least three independent experiments with triplicates in each experiment. ND, not determined.

<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>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>16.0 ± 0.9</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</td>
<td>8.2 ± 0.5</td>
<td>ND</td>
</tr>
<tr>
<td>9i</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</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.
proach 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 poly-valency 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. enriettii* cell line (LePentR50) to investigate the molecular mechanism of pentamidine resistance. First, the pentamidine resistance factor involved in the accumulation of pentamidine indirectly suggests that an efflux transporter is involved in pentamidine resistance in *L. enriettii* 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
The flavonoid dimers with three or four ethylene glycol units (9c or 9d) displayed the greatest modulatory activity, with ~3-fold sensitizations of pentamidine and SSG resistance, respectively, and in a dose-dependent manner. Moreover, their reversal activity on antileishmanial drug resistance was explained by the increase in intracellular accumulation of pentamidine and total antimony. Although the present study demonstrates that flavonoid dimers are effective modulators in vitro, animal experiments are required to determine whether the flavonoid dimers have the potential to be developed as an effective chemosensitizer for inhibiting pentamidine and SSG resistance in *Leishmania*.

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