Quinacrine and a novel apigenin dimer can synergistically increase the pentamidine susceptibility of the protozoan parasite *Leishmania*

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**Objectives:** The aim of this study was to investigate the synergistic effect of quinacrine and a novel apigenin dimer (compound 9d) on reversing pentamidine resistance of *Leishmania* parasites.

**Methods:** Pentamidine-resistant cell lines, LePentR50 and LdAG83PentR50, were generated by gradually increasing pentamidine pressure on wild-type promastigotes. We tested the effects of different combinations of quinacrine and an apigenin dimer on modulating the pentamidine resistance levels of LePentR50 and LdAG83PentR50 using an MTS proliferation assay. We then measured the accumulation level of pentamidine using HPLC. The fractional inhibitory concentration index (FICI) method was used to evaluate the interaction between quinacrine and the apigenin dimer on reversing pentamidine resistance in *Leishmania*.

**Results:** LePentR50 and LdAG83PentR50 promastigotes were ~8.6- and 4.6-fold more resistant to pentamidine than their wild-type parents. Amastigotes derived from LePentR50 and LdAG83PentR50 were also pentamidine-resistant. We found that quinacrine can increase the susceptibility of *Leishmania* to pentamidine. Quinacrine, when used at 6 μM, can increase the IC₅₀ of pentamidine by 3.8-, 3.4-, 3.5- and 6.3-fold in wild-type *Leishmania enriettii* Le, LePentR50, wild-type *Leishmania donovani* LdAG83 and LdAG83PentR50, respectively. Quinine, quinidine and verapamil did not show any sensitizing effect. The sensitizing effect of quinacrine was: (i) dose-dependent; (ii) not associated with an increase in pentamidine accumulation; and (iii) only observed in pentamidine-resistant but not sodium stibogluconate-resistant or vinblastine-resistant parasites. Other than quinacrine, we also found that an apigenin dimer (compound 9d), previously shown to be able to inhibit ABCB1-mediated cancer drug resistance in mammalian cells, can also increase the pentamidine susceptibility of *Leishmania*. 9d, when used at 6 μM, can increase the IC₅₀ of pentamidine by 2.5-, 4.2-, 1.6- and 1.9-fold in Le, LePentR50, LdAG83 and LdAG83PentR50, respectively. Unlike quinacrine, sensitization by 9d was accompanied by an increase in pentamidine accumulation, presumably due to the inhibition of an ABC transporter. Using the FICI method, we found that quinacrine and 9d can act synergistically. When they are used in a 1:1 ratio, they sensitize LePentR50 to pentamidine by 19-fold, with an FICI of 0.48 (P<0.005), indicating that they might act synergistically.

**Conclusions:** Our findings support the notion that the pentamidine susceptibility of *Leishmania* is mediated by multiple targets. Quinacrine and apigenin dimer 9d, each inhibiting its own target, can have a synergistic effect when used together to sensitize *Leishmania* to pentamidine.

Keywords: reversal of resistance, flavonoids, drug efflux

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Introduction

Leishmaniasis, one of the six major parasitic diseases targeted by the WHO, is endemic in 88 countries of the world, mainly in northern Africa, Asia, Latin America and the Middle East. A recent report from the WHO indicated that 350 million people are at risk of infection with *Leishmania* and that ~2 million are infected with leishmanial parasites annually, and 500,000 of these are visceral leishmaniasis, which could be fatal if left untreated. In the absence of effective vaccines and vector control, chemotherapy still plays a critical role in the control of the infection. Chemotherapy based on pentavalent antimonials (SBV) is the primary means for the treatment of leishmaniasis, and has been used for >50 years.

Recently, other therapeutic options to treat leishmaniasis have appeared promising. Paromomycin sulphate is best known for its use as a broad-spectrum aminoglycoside antibiotic. It acts as a topical antileishmaniasis agent. It is commonly used to treat Old World cutaneous leishmaniasis (*Leishmania major, Leishmania tropica, Leishmania aethiopica*). Paromomycin injection has been experimentally used to treat visceral leishmaniasis (primarily due to *Leishmania donovani*). The encouraging result of a Phase III clinical trial of paromomycin has led to the licensing of paromomycin to be used in treating visceral leishmaniasis in India. Miltefosine, a phosphocholine analogue, is also the first oral antileishmanial drug and its mode of action is probably through cell-signalling pathways and membrane synthesis. It has very promising results (cure rates >95%) in Indian visceral leishmaniasis and is undergoing clinical trials for use in several other countries.

Pentamidine, a synthetic derivative of amidine, has been used for >50 years in treating African trypanosomiasis. It also has a good clinical activity against *Leishmania*. It is used in the treatment of visceral leishmaniasis refractory to ShV treatments. It comprises the first-line treatment of cutaneous leishmaniasis in New Guyana where >90% of infections are due to *Leishmania guyanensis*. Pentamidine may inhibit many different cellular processes. In *L. donovani*, pentamidine was found to be a competitive inhibitor of arginine transport and a non-competitive inhibitor of putrescine and spermidine transport in *Leishmania infantum, L. donovani* and *Leishmania mexicana*. Cationic pentamidine may also preferentially bind to kinetoplast DNA and interfere with replication and transcription at the mitochondrial level. Pentamidine may target mitochondria in *Leishmania* because it induced structural changes to mitochondria. It may also affect the mitochondrial topoisomerase II of *Trypanosoma equiperdum in vivo*. The plasma membrane Ca²⁺-Mg²⁺-ATPase of *Trypanosoma brucei*, the S-adenosylmethionine decarboxylase of *T. brucei* and the mitochondrial membrane potential of *L. donovani*. Recently, resistance to pentamidine has been described in *trypanosomes* and *Leishmania*. Pentamidine-resistant *L. mexicana* and *L. donovani* accumulated less pentamidine than their wild-type counterparts. The lowered uptake of pentamidine may be due to a lowered mitochondrial membrane potential. Pentamidine resistance in *L. mexicana* was demonstrated to be associated with the exclusion of pentamidine from the mitochondrion. A functional complementation approach has also been used to identify a pentamidine resistance protein 1 (PRP1) that belongs to a member of the ATP-binding cassette (ABC) transporter superfamily in drug-selected *L. major*.

PRP1 has recently been localized to an intracellular compartment. In *Leishmania enriettii*, we have demonstrated that LeMDR1, an intracellular ABC transporter localized to internal organelles and connected to mitochondria in *L. enriettii*, was associated with pentamidine susceptibility. Furthermore, we have hypothesized that LeMDR1 is involved in the sequestration of pentamidine into mitochondria, thereby explaining why the LeMDR1 gene copy number is inversely related to pentamidine resistance. Recently, we have demonstrated that novel synthetic apigenin dimers of general structure 9, in particular 9d (Figure 1), were capable of reversing pentamidine resistance, presumably by inhibiting an ABC-type transporter.

Quinacrine is an acidrine derivative with antimalarial, antileishmanial and antitypanosomal activities. Various modes of action of quinacrine and its derivatives have previously been proposed, including inhibition of trypanothione reductase, intercalation and binding to haem and inhibition of enzyme topoisomerase II. In this study, we investigated the modulating effect of quinacrine on sensitizing *Leishmania* to pentamidine, and the possible synergistic effect of quinacrine and apigenin dimer 9d in the pentamidine susceptibility of *Leishmania*.

Materials and methods

Chemicals

Compound 9d was prepared according to published procedures. Quinacrine, quinine, quinidine, apigenin, vinblastine, verapamil and pentamidine were obtained from Sigma. Sodium stibogluconate (SSG) was a gift from GlaxoSmithKline.

Cell lines and cell culture

Promastigotes of *L. enriettii* (Le wild-type, LePentR50 and LeMDR1-overexpressing LeV160 mutants) and *L. donovani* (LdAG83, LdAG83PentR50 and Ld39) were employed in this study. *L. enriettii* is a natural infective strain of guinea pigs and *L. donovani* is a clinical strain, which mainly causes fatal visceral leishmaniasis in humans. Both strains were cultured in Schneider’s *Drosophila* Medium (Invitrogen), pH 6.9, supplemented with 10% (v/v) heat-inactivated fetal bovine serum (HyClone) with 4 mM glutamine (Sigma) and 36 μM gentamicin solution (Invitrogen), at 27°C for 4 days.

Promastigotes of LePentR50 (pentamidine-resistant, IC₅₀ of pentamidine = 224 μM) and LdAG83PentR50 (pentamidine-resistant, IC₅₀ of pentamidine = 74 μM) were selected by gradually increasing...
the pentamidine pressure on wild-type promastigotes. Promastigotes of Ld39 (SSG-resistant, IC50 of SSG = 7.1 mM) were cultured in the presence of 3.9 mM SSG. Promastigotes of LeV160 were cultured in the presence of 176 μM vinblastine.

Isolation of macrophages and in vitro infection of macrophages by promastigotes

Peritoneal macrophages were isolated from BALB/c mice. First, the BALB/c mice (4–6 weeks old) were treated intraperitoneally with 1 mL of 2% starch (Sigma) in water. After 3 days, peritoneal macrophages were harvested by peritoneal lavage using Dulbecco's modified Eagle medium (DMEM) (Invitrogen). Cells were counted and resuspended at a concentration of 2 × 10^6/mL in DMEM containing 10% heat-inactivated fetal bovine serum (Hyclone), 100 U/mL penicillin and 69 μM streptomycin (Hyclone). A round cover slip (12 mm in diameter) was placed into each well of a 24-well culture plate. Macrophages were seeded onto each well in a volume of 500 μL/well. Macrophages were allowed to attach overnight and the non-adherent cells were removed by gently washing twice with DMEM. The adherent macrophages were incubated with stationary-phase promastigotes at a parasite-to-macrophage ratio of 20:1 for 3h at 37°C with 5% CO2. Non-internalized promastigotes were removed by washing with DMEM three times. Infected macrophages were further incubated in 500 μL of DMEM containing 10% fetal bovine serum and antibiotics in the presence or absence of pentamidine for 48 h. After incubation, the coverslips were stained with Giemsa, and the amastigotes inside each macrophage (100 macrophages per treatment) were counted under a microscope and the percentage of macrophages infected and the number of amastigotes per 100 macrophages were enumerated.

Cell viability assay

The viability of promastigotes was determined by the Cell Titer 96® Aqueous Assay (Promega) that employs a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium; MTS] and electron coupling reagent, phenazine methosulphate (PMS).34 Promastigotes were seeded onto a 96-well flat-bottomed microtitre plate at 1 × 10^5 cells per well in a final volume of 100 μL of medium. To determine the modulating effects of apigenin, quinacrine, quinine, quinidine and verapamil, a concentration of 6 μM of the modulator was used in combination with various concentrations of antileishmanial drugs including pentamidine, SSG and vinblastine. To determine the dose dependence of quinacrine or 9d on increasing the pentamidine susceptibility of the parasites, various concentrations of quinacrine or 9d were added to the promastigotes as indicated in the figures. Parasites were incubated at 27°C for 72 h. Each concentration of antileishmanial was tested in triplicate, and repeated three times in separate experiments. A fresh solution of 4.1 mM MTS and 2.9 mM PMS was prepared at a ratio of 20:1 (MTS/PMS). After 72 h of incubation, 10 μL of the MTS/PMS mixture was added into each well of the microtitre plate. The plate was then incubated at 27°C for 4 h for colour development. After 4 h of incubation, the OD values were determined at 490 nm using an automatic microtitre plate reader (Bio-Rad). The results were presented as the percentage of survivors (OD value with test compound divided by that of untreated control). EC50 values can be obtained by plotting the IC50 values against the concentrations of modulator used in obtaining the corresponding IC50b. Curve fitting using non-linear regression was done using Prism 4.0 software.

Determination of in vitro synergistic activity

In vitro drug interaction between quinacrine and compound 9d on reversing pentamidine resistance was assessed using the fractional inhibitory concentration (FIC) index (FICI) method modified from Chou and Talalay.43 Promastigotes (LePentR50) were seeded onto a 96-well flat-bottomed microtitre plate at 1 × 10^5 cells per well in a final volume of 100 μL of medium. Seven different ratios of quinacrine and 9d were tested in triplicate, and repeated two times in separate experiments. A fresh solution of 4.1 mM MTS and 2.9 mM PMS was prepared at a ratio of 20:1 (MTS/PMS). After 72 h of incubation, the coverslips were stained with Giemsa, and the amastigotes inside each macrophage (100 macrophages per treatment) were counted under a microscope and the percentage of macrophages infected and the number of amastigotes per 100 macrophages were enumerated.

Pentamidine accumulation by HPLC

One milliliter of 4-day-old promastigotes (2 × 10^7 cells/mL) was washed with PBS twice and then incubated with 0.84 mM pentamidine and various concentrations of quinacrine or apigenin dimer 9d at 0, 15, 30 or 60 μM at 27°C for 3 h in the dark. Each concentration of quinacrine or 9d was tested in triplicate, and repeated two times in separate experiments. After 3 h of incubation, the parasites were washed three times with cold PBS, pH 7.4. The cell pellet was then dissolved in 350 μL of 75% acetonitrile and lysed by repeated freeze–thaw cycles. After lysing, the lysed cell suspension was centrifuged at 14000 g at 4°C for 10 min. The supernatant was collected and its pentamidine concentration was determined using HPLC (Agilent 1100 Series).27 The pentamidine pools were analysed on a Zorbax ODS C18 column (4.6 mm × 25 cm, 5 μm) kept at 40°C. The mobile phase consisted of 10 mM tetramethylammonium chloride (TMAC)/10 mM sodium heptanesulphonate (SHS)/4.2 mM phosphoric acid (PA) for pump A and 75% acetonitrile (ACN) in 10 mM TMAC/10 mM SHS/4.2 mM PA for pump B. The column was equilibrated at 40°C overnight before analyses. Using a flow rate of 1.0 mL/min and signal at 265 nm, analyses were made at 58% pump A and 42% pump B. The retention time of pentamidine was 3.2 min. Compound 9d could not be eluted out under these conditions. To generate a standard curve, a 200 μM stock solution of pentamidine isethionate salt was 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 made by serial dilution, allowing the generation of a standard curve.

Results

Characterization of pentamidine-resistant promastigotes and amastigotes

Two pentamidine-resistant promastigote cell lines of Leishmania were used in this study. They are LePentR50 and
LdAG83PentR50, obtained by applying increasing pentamidine pressure to wild-type *L. enriettii* (Le) and *L. donovani* (LdAG83) promastigotes, respectively. The IC50 (i.e. concentration of drug that kills 50% of cells) values for LePentR50 and Le were 228.6 and 26.6 μM, respectively, giving an ~8.6-fold increase in pentamidine resistance (Table 1). The IC50s of pentamidine for LdAG83PentR50 and LdAG83 were 74.7 and 16.2 μM, respectively, giving an ~4.6-fold increase in pentamidine resistance (Table 1).

We have also studied the pentamidine resistance of the intracellular amastigotes using the macrophage infection model. For both pentamidine-resistant strains (LePentR50 and LdAG83PentR50), the percentage of macrophages infected remained at ~87% and 78%, respectively, at the highest concentration of pentamidine used (33.7 μM) (Figure 2a). In contrast, the corresponding percentages for wild-type Le and LdAG83 dropped to ~36% and 39%, respectively, (Figure 2a) at the same concentration of pentamidine (33.7 μM). When we measured the number of amastigotes per 100 macrophages, we also found that this parameter was relatively unaffected in the pentamidine-resistant amastigotes of LePentR50 and LdAG83PentR50 when an increasing concentration of pentamidine was used (Figure 2b). In contrast, this parameter gradually decreased in both wild-type Le and LdAG83 when increasing concentrations of pentamidine were used (Figure 2b). These data suggest that the pentamidine resistance level can be maintained in the pentamidine-resistant amastigotes of LePentR50 and LdAG83PentR50.

**Quinacrine can sensitize Leishmania to pentamidine**

Overexpression of P-glycoprotein (P-gp) or multidrug resistance-associated protein 1 (MRP1) is a commonly observed mechanism by which tumour cells or parasites acquire multidrug resistance (MDR). It is possible that the pentamidine resistance in LePentR50 and LdAG83PentR50 was also mediated by either P-gp or MRP1. We therefore tested whether MDR modulators could inhibit the pentamidine resistance in LePentR50 and LdAG83PentR50 and was also mediated by either P-gp or MRP1. We therefore tested whether MDR modulators could inhibit the pentamidine resistance in LePentR50 and LdAG83PentR50. Here, a number of MDR modulators, including quinine, quinidine, verapamil and quinacrine, were tested for their ability to reverse pentamidine resistance. The concentration of 6 μM was chosen because quinacrine at such a concentration was relatively non-toxic to *Leishmania* (Table 2). The IC50 of quinacrine for Le, LePentR50, LdAG83 and LdAG83PentR50 were 18, 29, 12 and 12 μM, respectively (Table 2).

We found that quinacrine can sensitize both *L. enriettii* and *L. donovani* promastigotes to pentamidine, regardless of whether they are wild-type or pentamidine-resistant. Table 1 shows that quinacrine at 6 μM can reduce the IC50 of pentamidine from 2.66 to 7.1 μM in Le wild-type [relative fold (RF) = 3.8], from 228.6 to 67.8 μM in LePentR50 (RF = 3.4), from 16.2 to 4.6 μM in LdAG83 (RF = 3.5) and from 74.7 to 11.8 μM in LdAG83PentR50 (RF = 6.3) (Table 1). Such a sensitization effect was quinacrine dose-dependent. When the concentration of quinacrine was decreased gradually from 6 to 0.375 μM, there was a corresponding decrease in the susceptibility to pentamidine in all four *Leishmania* cell lines (Table 1). The EC50 values of quinacrine (effective concentration that can decrease the IC50 of pentamidine by 50%) for Le wild-type, LePentR50, LdAG83 and LdAG83PentR50 have been determined to be 1.8, 3.3, 0.7 and 0.6 μM, respectively.

Quinacrine, however, has no sensitization effect on either SSG-resistant *L. donovani* Ld39 or vinblastine-resistant *L. enriettii* LeV160 known to contain amplified copies of *LeMDR1* (Table 1). Other MDR modulators, including quinine, quinidine and verapamil, when used at 6 μM, did not show any significant sensitization effect on pentamidine, SSG or vinblastine (Table 1).

**Quinacrine increases the pentamidine susceptibility without affecting pentamidine accumulation**

We are interested in understanding the mechanism of pentamidine resistance in LePentR50 and LdAG83PentR50. The effect of quinacrine on the accumulation of pentamidine was investigated. Here, a higher concentration of quinacrine (ranging from 15 to 60 μM) was used compared with that in the cytotoxicity assay (as low as 1 μM). A higher concentration was used because a shorter incubation time of 3 h was used in the accumulation assay. We found that there was no significant difference in pentamidine accumulation between wild-type (Le and LdAG83) and pentamidine-resistant counterparts (LePentR50 and LdAG83PentR50) (Figure 3a). Quinacrine did not show any significant effect on the intracellular pentamidine concentration in either susceptible (Le and LdAG83) or resistant *Leishmania* (LePentR50 and LdAG83PentR50) (Figure 3a). This result suggests that: (i) lowered accumulation of pentamidine was not the mechanism of resistance in LePentR50 and LdAG83PentR50; and (ii) the mechanism by which quinacrine increased the pentamidine susceptibility of parasites was not due to an increase in pentamidine accumulation. This result suggests that the target of quinacrine may not be a pentamidine transporter on the plasma membrane. Parasites incubated with the same concentrations of quinacrine for the same period of time did not show any apparent cytotoxicity, suggesting that the lack of pentamidine efflux was not due to quinacrine cytotoxicity (Figure 3b).

**Apigenin dimer 9d increases the pentamidine susceptibility in a dose-dependent manner by increasing pentamidine accumulation**

We have previously demonstrated that synthetic apigenin dimers of general structure 9 (Figure 1), particularly compound 9d, can modulate pentamidine and SSG resistance in *Leishmania*. 9d is a synthetic apigenin dimer made up of two apigenin moieties linked together by a linker consisting of four ethylene glycol units. We have demonstrated that 9d is a specific modulator of P-gp in mammalian cancer cells and *Leishmania*. Here, we studied the effect of 9d on the pentamidine susceptibility of Le wild-type, LePentR50, LdAG83 and LdAG83PentR50. The apigenin dimer 9d was found to be non-toxic to Le wild-type and LePentR50 even at 200 μM (Table 2). On the other hand, 9d was toxic to LdAG83 and LdAG83PentR50 and their IC50 were 7 and 5 μM, respectively (Table 2). For easy comparison, the starting concentration of quinacrine and 9d was 6 μM and a serial dilution was carried out to observe their effects on reversing pentamidine resistance of both *L. enriettii* and *L. donovani*.

Similarly to quinacrine, 9d demonstrated a dose-dependent effect on increasing the pentamidine susceptibility, particularly of Le wild-type and LePentR50 (Table 1). RF values for Le,
Table 1. Modulatory activity of MDR modulators on susceptibility to pentamidine, SSG and vinblastine

<table>
<thead>
<tr>
<th>Modulators</th>
<th>IC_{50} of pentamidine in μM (RF)</th>
<th>IC_{50} of SSG in mM (RF)</th>
<th>IC_{50} of vinblastine in μM (RF)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Le wild-type</td>
<td>LdPentR50</td>
<td>LdAG83</td>
</tr>
<tr>
<td>None</td>
<td>26.6 ± 0.5 (1.0)</td>
<td>228.6 ± 0.8 (1.0)</td>
<td>16.2 ± 0.3 (1.0)</td>
</tr>
<tr>
<td>Quinine (6.0 μM)</td>
<td>25.5 ± 0.2 (1.0)</td>
<td>186.4 ± 0.8 (1.2)</td>
<td>25.3 ± 0.2 (0.6)</td>
</tr>
<tr>
<td>Quinidine (6.0 μM)</td>
<td>25.8 ± 1.3 (1.0)</td>
<td>168.8 ± 5.1 (1.4)</td>
<td>16.0 ± 0.8 (1.0)</td>
</tr>
<tr>
<td>Verapamil (6.0 μM)</td>
<td>19.0 ± 0.5 (1.4)</td>
<td>144.3 ± 0.8 (1.6)</td>
<td>18.4 ± 2.5 (0.9)</td>
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<tr>
<td>Quinacrine (6.0 μM)</td>
<td>7.1 ± 0.2 (3.8)</td>
<td>67.8 ± 2.5 (3.4)</td>
<td>4.6 ± 0.3 (3.5)</td>
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<td>Quinacrine (3.0 μM)</td>
<td>9.1 ± 0.5 (2.9)</td>
<td>121.6 ± 2.5 (1.9)</td>
<td>2.7 ± 0.2 (6.0)</td>
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<tr>
<td>Quinacrine (1.5 μM)</td>
<td>14.3 ± 0.3 (1.9)</td>
<td>165.2 ± 1.7 (1.4)</td>
<td>4.7 ± 0.2 (3.4)</td>
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<tr>
<td>Quinacrine (0.75 μM)</td>
<td>15.4 ± 0.3 (1.7)</td>
<td>191.2 ± 1.7 (1.2)</td>
<td>7.1 ± 1.0 (2.3)</td>
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<tr>
<td>Quinacrine (0.375 μM)</td>
<td>16.2 ± 0.3 (1.6)</td>
<td>214.4 ± 0.8 (1.1)</td>
<td>10.4 ± 0.7 (1.6)</td>
</tr>
<tr>
<td>9d (6.0 μM)</td>
<td>10.4 ± 0.7 (2.5)</td>
<td>54.0 ± 0.5 (4.2)</td>
<td>10.1 ± 0.8 (1.6)</td>
</tr>
<tr>
<td>9d (3.0 μM)</td>
<td>12.7 ± 1.2 (2.1)</td>
<td>79.3 ± 1.2 (2.9)</td>
<td>7.4 ± 0.8 (2.2)</td>
</tr>
<tr>
<td>9d (1.5 μM)</td>
<td>17.0 ± 1.7 (1.6)</td>
<td>89.4 ± 1.7 (2.6)</td>
<td>9.3 ± 1.5 (1.7)</td>
</tr>
<tr>
<td>9d (0.75 μM)</td>
<td>24.0 ± 1.7 (1.1)</td>
<td>128.2 ± 1.7 (1.8)</td>
<td>8.8 ± 1.2 (1.8)</td>
</tr>
<tr>
<td>9d (0.375 μM)</td>
<td>27.0 ± 3.2 (1.0)</td>
<td>175.5 ± 6.7 (1.3)</td>
<td>11.8 ± 1.7 (1.4)</td>
</tr>
<tr>
<td>Apigenin (6.0 μM)</td>
<td>22.6 ± 0.7 (1.2)</td>
<td>146.8 ± 1.7 (1.6)</td>
<td>15.0 ± 1.9 (1.1)</td>
</tr>
</tbody>
</table>

ND, not done; RF, relative fold \([= \text{IC}_{50} \text{ of pentamidine (or SSG or vinblastine)} \text{ in the absence of modulator divided by the IC}_{50} \text{ of the same drug with modulator}]\).

The modulatory activity of different modulators was studied by determining the IC_{50} values. Each concentration of drug with or without modulators was tested in triplicate and repeated at least twice in separate experiments. All values are displayed as mean ± SEM from two independent experiments.
LePentR50, LdAG83 and LdAG83PentR50 (using 6 mM 9d) were 2.5, 4.2, 1.6 and 1.9, respectively. The EC$_{50}$s for sensitizing the pentamidine susceptibility of Le wild-type and LePentR50 were 1.2 and 1.0 mM, respectively. It is important to note that the apigenin monomer did not show any significant modulating effect, implicating the importance of the dimeric structure of the apigenin dimer 9d in mediating the sensitization effect (Table 1).

We investigated the mechanism by which 9d increased the pentamidine susceptibility of Le and LePentR50 by measuring the effect of 9d on pentamidine accumulation. Here, a higher concentration of compound 9d (ranging from 15 to 60 mM) was used compared with that in the cytotoxicity assay (as low as 1 mM). A higher concentration was used because a shorter incubation time of 3 h was used in the accumulation assay. We found that 9d can increase pentamidine accumulation in both Le wild-type and LePentR50 in a dose-dependent manner (Figure 4a). Treatment of Le wild-type with 60 mM 9d can increase pentamidine accumulation from 2.5 to 27 mM/mg of protein (11-fold increase). We have reported similar results for LePentR50 where 60 mM 9d can increase pentamidine accumulation of LePentR50 cells from 2.0 to 27 mM/mg of protein (14-fold increase) (Figure 4a). 34 This result suggests that 9d may be modulating a pentamidine efflux transporter in both Le and LePentR50. Incubation of Le wild-type and LePentR50 with the same concentrations of 9d for the same period of time did not result in any significant cytotoxicity (Figure 4b). The dose-dependent increase in pentamidine accumulation was, therefore, note that the apigenin monomer did not show any significant modulating effect, implicating the importance of the dimeric structure of the apigenin dimer 9d in mediating the sensitization effect (Table 1).

We investigated the mechanism by which 9d increased the pentamidine susceptibility of Le and LePentR50 by measuring the effect of 9d on pentamidine accumulation. Here, a higher concentration of compound 9d (ranging from 15 to 60 mM) was used compared with that in the cytotoxicity assay (as low as 1 mM). A higher concentration was used because a shorter incubation time of 3 h was used in the accumulation assay. We found that 9d can increase pentamidine accumulation in both Le wild-type and LePentR50 in a dose-dependent manner (Figure 4a). Treatment of Le wild-type with 60 mM 9d can increase pentamidine accumulation from 2.5 to 27 mM/mg of protein (11-fold increase). We have reported similar results for LePentR50 where 60 mM 9d can increase pentamidine accumulation of LePentR50 cells from 2.0 to 27 mM/mg of protein (14-fold increase) (Figure 4a). 34 This result suggests that 9d may be modulating a pentamidine efflux transporter in both Le and LePentR50. Incubation of Le wild-type and LePentR50 with the same concentrations of 9d for the same period of time did not result in any significant cytotoxicity (Figure 4b). The dose-dependent increase in pentamidine accumulation was, therefore,

![Figure 2](https://example.com/figure2.png)
due to the modulatory effect of 9d, and not due to a cytotoxic effect on Le wild-type or LePentR50.

**Synergistic effect of quinacrine and 9d on increasing the pentamidine susceptibility of LePentR50**

These results suggest that quinacrine and 9d may be sensitizing the pentamidine susceptibility of Leishmania using different mechanisms. Quinacrine cannot increase pentamidine accumulation whereas 9d can. Here, we found that quinacrine and 9d have a synergistic effect on increasing the pentamidine susceptibility of LePentR50. We treated LePentR50 with different combinations of the two modulators quinacrine and 9d and determined their effects on the IC50 of pentamidine; 6 μM quinacrine or 6 μM 9d alone caused ~3.7-fold (IC50 = 44.5 μM) and 5.6-fold (IC50 = 29.4 μM) sensitization, respectively (Table 3). When we combined 3 μM 9d with 3 μM quinacrine, we observed a very strong sensitization effect (IC50 of pentamidine = 8.8 μM; 18.7-fold sensitization), suggesting that quinacrine and 9d can act synergistically (Table 3). Slightly less synergistic effect was observed when quinacrine and 9d were combined in ratios of 4:2 (IC50 of pentamidine = 10.8 μM; 15.1-fold sensitization) and 2:4 (IC50 of pentamidine = 13.7 μM; 12.0-fold sensitization) (Table 3). No apparent cytotoxicity was observed when we treated LePentR50 cells with quinacrine and 9d but without pentamidine, suggesting that the synergistic activity of quinacrine and 9d on reversing the pentamidine resistance of LePentR50 is not due to a cytotoxic effect of quinacrine and 9d (Figure 5).

To quantitatively measure the synergistic effect between quinacrine and 9d, we used the FICI approach (see the Materials and methods section for details) modified from Chou and Talalay.43 Briefly, we first determined the fractional IC50 (FIC) for quinacrine and 9d for each combination of modulators. The sum of two FICs will give the FICI value for that combination. An FICI value <0.5 indicates synergy. As shown in Table 4, the synergistic effect (FICI = 0.48; P < 0.005) was only observed in the combination of 3 μM quinacrine and 3 μM 9d. Other ratios were found to give an additive effect because their FICI values were between 0.6 and 1.1. Therefore, the combination ratio is very important for determining the degree of synergy.
Each concentration of 9d was tested in triplicate and repeated twice in separate experiments. The percentage of survivors was determined by MTS proliferation assay (mean ± SEM, n=2).

**Figure 4.** Effect of apigenin dimer 9d on pentamidine accumulation in *Leishmania* parasites. (a) Determination of pentamidine accumulation. One millilitre of 4-day-old promastigotes (Le wild-type and LePentR50) at a cell density of 2×10⁷ cells/mL was used for the pentamidine accumulation assay. The cells were washed with PBS and then incubated at 27°C for 3 h with increasing concentrations of 9d (0, 15, 30 and 60 μM). Each concentration of 9d was tested in triplicate and repeated twice in separate experiments. The amount of pentamidine accumulated was determined by HPLC and is presented as μM pentamidine per mg of protein (mean ± SEM, n=2). #Dose-dependent pentamidine accumulation in LePentR50 has been reported previously and is included in this figure for comparison. (b) Cytotoxic effect of 9d on *Leishmania* parasites. Promastigotes were treated as in (a). Each concentration of 9d was tested in triplicate and repeated twice in separate experiments. The percentage of survivors was determined by MTS proliferation assay (mean ± SEM, n=2).

Other than using the IC₅₀ to calculate the FICI, we also determined the IC₁₀, IC₃₀, IC₇₀ and IC₉₀ of pentamidine for LePentR50 cells for each of the quinacrine/9d combinations and used them to calculate the FICI. These FICI values were then plotted against Fₐ (Figure 6a; Fₐ=FICI plot). FICI values ≤0.5 suggest synergy. The strongest synergism (the lowest FICI) was observed at the ratio of 3:3. Figure 6(a) shows that FICI values are <0.5 at the Fₐ values of 0.5, 0.7 and 0.9 when quinacrine and 9d were used at a ratio of 3:3. An additive effect was observed when quinacrine and 9d were used at ratios of 1:5, 2:4, 4:2 and 5:1 (FICI values >0.5 and close to 1) (Figure 6a). This result suggests that quinacrine and 9d at a ratio of 3:3 are acting synergistically to sensitize *Leishmania* to pentamidine.

We also investigated the effect of quinacrine/9d on pentamidine accumulation in LePentR50 cells (Figure 6b). 9d, when used at 60 μM, resulted in a 12.1-fold increase in pentamidine accumulation whereas quinacrine, used at 60 μM, had no significant effect (1.2-fold increase). When 9d and quinacrine were used together in a ratio of 1:1 (30 μM quinacrine +30 μM 9d), we found that there was no synergism (3.0-fold) observed in terms of pentamidine accumulation (Figure 6b). This increase in pentamidine accumulation was only marginally increased over that of 30 μM 9d treatment alone (2.4-fold increase). This result suggested that the synergistic effect of 9d and quinacrine in increasing the pentamidine susceptibility was due neither to a potentiation of 9d nor an increase in pentamidine accumulation. This result further confirmed that 9d and quinacrine were targeting different mechanisms.

**Table 3.** Modulatory activity of different combinations of quinacrine and 9d on reversing pentamidine resistance of LePentR50

<table>
<thead>
<tr>
<th>Modulators used</th>
<th>IC₅₀ of pentamidine (μM) (RF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 μM quinacrine +0 μM 9d</td>
<td>163.7±15.7 (1.0)</td>
</tr>
<tr>
<td>6 μM quinacrine +0 μM 9d</td>
<td>44.5±13.0 (3.7)</td>
</tr>
<tr>
<td>5 μM quinacrine +1 μM 9d</td>
<td>17.5±3.2 (9.3)</td>
</tr>
<tr>
<td>4 μM quinacrine +2 μM 9d</td>
<td>10.8±2.9 (15.1)</td>
</tr>
<tr>
<td>3 μM quinacrine +3 μM 9d</td>
<td>8.8±2.7 (18.7)</td>
</tr>
<tr>
<td>2 μM quinacrine +4 μM 9d</td>
<td>13.7±2.7 (12.0)</td>
</tr>
<tr>
<td>1 μM quinacrine +5 μM 9d</td>
<td>18.4±2.9 (8.9)</td>
</tr>
<tr>
<td>0 μM quinacrine +6 μM 9d</td>
<td>29.4±4.2 (5.6)</td>
</tr>
</tbody>
</table>

RF, relative fold (IC₅₀ of pentamidine in the absence of modulators divided by the IC₅₀ of pentamidine in the presence of different combinations of quinacrine and 9d).
All values are displayed as mean±SEM from six independent experiments. The P value of IC₅₀ at different combinations of quinacrine and 9d when compared with 6 μM quinacrine alone or 6 μM 9d alone was determined by Student’s t-test.
*P<0.05.
**P<0.01.

**Discussion**

Cationic diamidines like pentamidine have a long history as valuable chemotherapeutic agents against infectious diseases. The mode of action of pentamidine is reported to be one that disrupts the parasite mitochondrion. Resistance to pentamidine has been observed in *Leishmania*. It is generally believed that reduced accumulation of pentamidine in
mitochondria and/or increased efflux was the basis of pentamidine resistance. To date, there is no effective way to overcome pentamidine resistance in *Leishmania*. The present work represents the first investigation into the modulatory activity of quinacrine on the pentamidine susceptibility of *Leishmania*. Quinacrine does not increase the pentamidine susceptibility by increasing pentamidine uptake, which distinguishes it from another pentamidine-resistance reversing agent (a synthetic apigenin dimer, 9d) we reported previously. More significantly, when these two mechanistically different modulating agents were combined, they gave a synergistic effect in increasing the pentamidine susceptibility. Quinacrine is an acridine derivative. It has multicellular targets and is likely to have multiple mechanisms of action. It can act on mitochondria of hepatocytes and beef heart, e.g. by decreasing the transmembrane potential of mitochondria and interacting with submitochondrial membranes. Compared with other known MDR modulators, including apigenin, quinine, quinidine and verapamil, we found that only quinacrine can restore the susceptibility of parasites to pentamidine (Table 1). Moreover, it is effective only in the pentamidine-resistant parasite, not in SSG- or vinblastine-resistant parasites (Table 1). Surprisingly, quinacrine not only restored the susceptibility of resistant parasites (LePentR50 and LdAG83PentR50), but also increased the susceptibility of wild-type parasites (Le wild-type and LdAG83). This result suggests that the quinacrine target remains unaltered in wild-type and pentamidine-resistant parasites. Whatever the quinacrine target might be, it cannot be the mechanism of pentamidine resistance in resistant strains.

Although our results clearly demonstrated the role of quinacrine in increasing the pentamidine susceptibility, we do not know how such sensitization works. We found that quinacrine did not increase pentamidine accumulation in *Leishmania* (Figure 3a). Interestingly, apigenin dimer can sensitize both Le wild-type and LePentR50 by increasing the intracellular accumulation of pentamidine and LePentR50 by increasing the intracellular accumulation of pentamidine. Previous studies have demonstrated that 9d can modulate ABC transporters in mammalian cancer cells and *Leishmania*. We have demonstrated that 9d can modulate ABC transporters in mammalian cancer cells and *Leishmania*. Our monoclinic green fluorescent protein (mGFP) reporter strain was used to monitor the intracellular accumulation of pentamidine. We observed a dose-dependent increase in mGFP fluorescence in both the wild-type and LePentR50 strains treated with 9d. However, we did not observe a similar increase in mGFP fluorescence when quinacrine was used. These results suggest that quinacrine can either be used to potentiate the cytotoxic effect of pentamidine in wild-type *Leishmania* or to reverse pentamidine resistance in resistant strains.

**Table 4. In vitro synergistic interaction of quinacrine and 9d on reversing pentamidine resistance of LePentR50**

<table>
<thead>
<tr>
<th>Conc. of quinacrine (μM)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc. of 9d (μM)</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Mean FIC (quinacrine)</td>
<td>1.0 ± 0.0</td>
<td>0.46 ± 0.06</td>
<td>0.26 ± 0.05</td>
<td>0.20 ± 0.06</td>
<td>0.36 ± 0.05</td>
<td>0.80 ± 0.07</td>
<td>1.0 ± 0.00</td>
</tr>
<tr>
<td>Mean FIC (9d)</td>
<td>0.0 ± 0.0</td>
<td>0.59 ± 0.07</td>
<td>0.35 ± 0.06</td>
<td>0.28 ± 0.07</td>
<td>0.46 ± 0.05</td>
<td>0.63 ± 0.04</td>
<td>1.0 ± 0.00</td>
</tr>
<tr>
<td>Mean FICI</td>
<td>1.0 ± 0.00</td>
<td>1.05 ± 0.11</td>
<td>0.61 ± 0.11</td>
<td>0.48 ± 0.12</td>
<td>0.82 ± 0.10</td>
<td>1.13 ± 0.08</td>
<td>1.0 ± 0.00</td>
</tr>
<tr>
<td>Drug interaction</td>
<td>—</td>
<td>additive</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

FIC values are calculated using the IC_{50} values from Table 3 and are presented as mean ± SEM from six independent experiments. The *P* values of FICIs at different combinations of quinacrine and 9d when compared with 6 μM quinacrine alone or 6 μM 9d alone were determined by Student's *t*-test. Interaction is classified as synergistic (FICI < 0.5), additive (FICI > 0.5–4) or antagonistic (FICI > 4). *a* | *p* < 0.005. *b* | *p* < 0.001. *c* | *p* < 0.01. *d* | *p* < 0.05.
Together with this report, we hypothesize that pentamidine may be exported in *Leishmania* by an ABC transporter, and this ABC transporter can be inhibited by 9d. Interestingly, 9d can sensitize both Le and LePentR50 to pentamidine to a similar extent (3.8- and 3.4-fold, respectively, for Le wild-type and LePentR50; Table 1). This same level of sensitization was reflected in the pentamidine accumulation (Figure 4a). These results suggest that the target of 9d, presumably an ABC transporter, is present at the same level in both Le and LePentR50. This explains why 9d can sensitize both Le and LePentR50 and increase their pentamidine accumulation to the same extent. The putative ABC transporter is also predicted not to be responsible for pentamidine resistance because it is present in the same amount in both Le and LePentR50.

Although neither the quinacrine target nor the 9d target (presumably an ABC transporter) in *L. enriettii* was responsible for the pentamidine resistance in LePentR50, our results demonstrated that these two mechanisms were independent of each other. Inhibition of the quinacrine target did not result in an increase in pentamidine accumulation, whereas inhibition of the 9d target did. Moreover, quinacrine and 9d have a synergistic effect on sensitizing LePentR50 to pentamidine (Tables 3 and 4 and Figure 6a), suggesting that a combination of these two modulators may be a promising way to overcome pentamidine resistance in *Leishmania* parasites. It would be worthwhile to further study the action of these two modulators in the hope that they can give us some insights into the mechanism of pentamidine resistance in *Leishmania*.

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**Transparency declarations**

None to declare.
Synergistic modulation of pentamidine resistance in *Leishmania*

References


