In vitro and *in vivo* efficacy of novel flavonoid dimers against cutaneous leishmaniasis

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Abbreviations: cutaneous leishmaniasis (CL), polyethyleneglycol (PEG), ethyleneglycol (EG), peritoneal elicited macrophage (PEM), sodium stibogluconate (SSG).

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ABSTRACT

Treatment of leishmaniasis by chemotherapy remains a challenge because of limited efficacy, toxic side effects and drug resistance. We have previously reported that synthetic flavonoid dimers have potent anti-promastigote and anti-amastigote activity against *Leishmania donovani*, causative agent of visceral leishmaniasis. Here we further investigate their leishmanicidal activities against cutaneous *Leishmania* strains. One of the flavonoid dimers (compound **39)** has a marked anti-promastigote (IC_{50} = 0.19 – 0.69 µM) and anti-amastigote (0.17 – 2.2 µM) activities towards different species of *Leishmania* that cause cutaneous leishmaniasis (CL), including *L. amazonensis, L. braziliensis, L. tropica* and *L. major*. Compound **39** is not toxic to peritoneal elicited macrophages with IC_{50} higher than 88 µM. In the mouse model of cutaneous leishmaniasis induced by subcutaneous inoculation of *L. amazonensis* in mouse foot pad, intralesional administration of 2.5 mg/kg of **39.HCl** can reduce foot pad thickness by 36% compared to control. Amastigotes loading lesion was reduced by 20-fold. The present study suggests that flavonoid dimer **39** represents a new class of safe and effective leishmanicidal agent against both visceral and cutaneous leishmaniasis.

Introduction

Leishmaniasis is a serious parasitic disease found in 98 countries spread in 5 continents with an annual death of 20,000 to 40,000 (1). The disease has three main types of manifestations: visceral, cutaneous and mucocutaneous. Cutaneous leishmaniasis (CL) has an annual occurrence of 0.7 to 1.2 million (1). The most-affected countries include Afghanistan, Algeria, Iran and Brazil. CL usually induces skin ulcers on the exposed parts of the body. If left untreated, CL leaves permanent scars and may cause body disfiguration. This disease is widespread in both Old and New World (16). Currently, there is no human vaccine available and chemotherapy is the major approach for treatment of leishmaniasis (10). Pentavalent antimonial compounds like Pentostam or Glucantime are first-line antileishmanials that have been used clinically for over 50 years (2, 20). Antimonial treatment, however, is far from satisfactory due to the need for intramuscular administration and long treatment time, side effects and emergence of antimonials-resistant cases (8, 14, 20). Second-line drugs, such as amphotericin B and pentamidine, may be toxic and expensive (2). The new oral drug, miltefosine, is highly efficacious, but suffers from problems like the need for long treatment time, potential teratogenicity and long residence time in patients which can potentially lead to drug resistance. There is an urgent need for novel, cheaper, potent and safe antileishmanial compounds for treating leishmaniasis.

Plant-derived natural products such as flavonoids have been reported to have a wide range of biological activities like antioxidation and anticancer (11, 13). They have been a good source for discovering new anti-leishmanial agents (12, 17, 19). Previously, we demonstrated that synthetic flavonoid dimers can inhibit the pumping activity of ATP-binding Cassette (ABC) transporters, resulting in an increase in intracellular drug accumulation and thereby reversing drug resistance in both cancer and *Leishmania* (3-5, 23, 25, 26). Furthermore, some of the flavonoid dimers were found to have potent anti-promastigote and anti-amastigote activity towards *L. donovani* which causes fatal visceral form of leishmaniasis. IC_{50} was around 0.2 μM for promastigotes and 0.6 μM for amastigotes (24).

In the present study, we further demonstrate that flavonoid dimers have significant antileishmanial activity against several species of *Leishmania* which cause CL *in vitro.* For one particularly active compound, flavonoid dimer **39**, its anti-promastigote and anti-amastigote activities were studied and compared with known antileishmanial agents in terms

of their therapeutic index. Finally, the *in vivo* efficacy of **39.HCl** against cutaneous leishmaniasis in a mouse model was also demonstrated.

MATERIALS AND METHODS

Chemicals. The flavonoid dimers **1-3**, **5-30** and amino-ethyleneglycol-linked flavonoid dimers **31-50**, **53**, **56-61** and **68-73** were prepared according to the reported procedures and their chemical structures were shown in Chart 1 (3-5, 24). **39.HCl** salt was prepared by adding excess 35% concentrated hydrochloric acid to a solution of **39** in chloroform. After stirring for 30 minutes, the mixture was evaporated to dryness under high vacuum to afford **39.HCl** salt as brownish solid. The purity of tested compounds was determined by HPLC, which was performed by using an Agilent 1100 series installed with an analytic column of Agilent Prep-Sil Scalar column (4.6 mm × 250 mm, 5 μm) at UV detection of 320 nm (reference at 450 nm) with isocratic elution of hexane (50%)/ethyl acetate (25%)/methanol (25%) at a flow rate of 1 mL/min. All tested compounds were shown to have > 95% purity according to HPLC. Pentamidine and amphotericin B were purchased from Sigma. Miltefosine was from Cayman (Michigan, USA). Sodium stibogluconate (SSG) was a generous gift from Glaxo Smith Kline (UK).

Cell lines and Cell Culture. Promastigotes of cutaneous *Leishmania* (*L. major* 50122 [MHOM/IL/67/JERICHO II (Lm50122), from ATCC], *L. amazonensis* [*L. amazonensis* LV78], *L. braziliensis* [*L. braziliensis* UA847], *L. major Friedlin* [*L. major* FV1] and *L. tropica* EP41 were employed in this study. The last four strains were kindly provided by Prof. K. P. Chang. All strains were cultured in Schneider's *Drosophila* Medium (Invitrogen, USA), pH 6.9 supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS) (Hyclone) with 4 mM glutamine (Sigma) and 25 μg/mL gentamicin solution (Invitrogen) at 27^{o}C for 4 days (6).

***In vitro* anti-promastigote activity.** Anti-promastigote activity was determined according to previous procedures (23) by Cell Titer 96$^{®}$Aqueous Assay (Promega) that employed a tetrazolium compound. Promastigotes were seeded into 96-well flat bottom microtiter plate at 1 x 10^5 cells per well in a final volume of 100 μL medium and incubated with a series of concentrations of synthetic flavonoid dimers or known antileishmanials. Parasites were incubated at 27^{o}C for 72 hours. After 72 hours of incubation, 10 μL of MTS:PMS mixture [MTS:2-(4,5-Dimethylthiazol-2-yl-)-5-[3-(carboxymethoxy)phenyl]-2-(4-sulfophenyl)-2H-

tetra- zolium); PMS: phenazine methosulfate both purchased from Sigma] was added into each well of microtiter plate. The plate was then incubated at 27ºC for 4 hours for color development. After 4 hours of incubation, the OD values were determined at 490 nm using automatic microtiter plate reader (Bio-Rad).

***In vitro* anti-amastigote activity.** Mouse peritoneal elicited macrophages (PEM) were obtained as previously described (26). A round cover slip (12 mm in diameter) was placed into each well of 24-well culture plate. Mouse PEM were resuspended in supplemented DMEM media containing 10% heat inactivated FBS (v/v), 100 U/mL penicillin and 100 µg/mL streptomycin and seeded into each well at a cell density of $1x10^5$ cells per 500 µL. Macrophages were allowed to attach overnight. Non-adherent cells were removed by gentle washing with un-supplemented DMEM media twice. Adherent macrophages were infected with late-log promastigotes at a parasite-to-macrophage ratio of 20:1 overnight at 37ºC with 5% CO_2. Non-internalized promastigotes were removed by washing twice with un-supplemented DMEM media. Infected macrophages were further incubated in 500 µL of supplemented DMEM media in the presence or absence of flavonoid dimers or known antileishmanials for 72 hours at 37ºC. After incubation, cover slips were stained with Giemsa and the percentage of macrophages infected and number of amastigotes per 100 macrophages was enumerated.

***In vivo* cutaneous leishmaniasis mouse model.** CL animal model was established using highly susceptible 3-4 week old female Balb/c mice weighing about 20 grams. Animals were housed in groups of five in cages in controlled environmental conditions (12:12 hr light/dark cycle and room temperature).

On day 0, Balb/c mice were infected by subcutaneous inoculation of $1x10^7$ infective promastigotes of *L. amazonensis* LV78 into the left hind of footpad. Thickness of footpad lesion was measured using dial caliper. Treatments were initiated when the lesion thickness reached approximately 0.5 mm on day 16 post-infection. 50 µL of either antileishmanials or control solvent was administered intralesionally every 4 days. The treatment groups received either 0.5 or 2.5 mg/kg of **39.HCl** dissolved in 50% polyethylene glycol (PEG) (n=12 mice). Positive control received 28 mg/kg SSG dissolved in 0.9% NaCl (n=11 mice). The solvent control received 50% PEG (n=11 mice). The untreated control received 0.9% NaCl (n=5 mice). A total of eight injections on days 16, 20, 24, 28, 32, 36, 40 and 44 were made. The experiment was completed on day 48. Lesion size and weight of mice were measured after

each treatment throughout the experiment. Lesion size was determined by subtracting the thickness of left lesion-bearing footpad from the right uninfected control footpad. All mice from different groups were then sacrificed to determine lesion weight and parasite burden.

All animals used in this work were conducted using the guidelines provided by the Laboratory Animal Use and Care from the Chinese CDC and the Rules for Medical Laboratory Animals (1998) from the Ministry of Health, China. All protocols for animal use in this work were approved by the Laboratory Animal Use and Care Committee of Sun Yat-Sen University under the license number 2012CB53000.

Parasite burden by limiting dilution assay. On 48 days post-infection, four mice from each group were picked randomly to assess the parasite number in each lesion. Parasite number in lesion was determined by limiting dilution assay. Under sterile condition, lesion-bearing footpad was removed, cut into small pieces and re-suspended in 5 mL of supplemented Schneider's *Drosophila* media. It was homogenized in a glass homogenizer by pulling upwards and downwards for 10 times. The cell suspensions were serially diluted in a 10-fold dilution (from 1:10 to $1:1x10^7$). 1.2 mL of the diluted suspension was aliquoted into 96-well plate, with 100 µL per well. The plate was incubated at 27^{o}C for 2 weeks and then examined under inverted microscope to determine the presence or absence of mobile promastigotes. Final titer was defined as the last dilution at which at least one well contained no parasite.

RESULTS

***In vitro* anti-promastigote activity**. In our previous study, we reported the anti-promastigote activity of flavonoid dimers toward different strains of *L. donovani* promastigotes and amastigotes, including wild-type LdAG83, sodium stibogluconate (SSG)-resistant Ld39 and pentamidine-resistant LdAG83PentR50 (18). In this study, five strains of CL-causing *Leishmania* species including *L. amazonensis* LV78, *L. braziliensis* UA847, *L. tropica* EP41, *L. major* FV1 and *L. major* Lm50122 were studied. Flavonoid dimers **1-3**, **5-30** and amino-ethyleneglycol-linked flavonoid dimers **31-50**, **53**, **56-61** and **68-73** were prepared according to the reported procedures and their chemical structures were shown in Chart 1 (3-5, 24).

*(A) Polyethylene glycol (PEG) linked flavonoid dimers **1-3** and **5-30***

All flavonoid dimers in series **A** contain OH groups in the ring A or ring C with different linker lengths from 3 to 5 EG units (Chart 1). Compounds **1** and **2**, with 3 to 4 EG units, displayed anti-promastigote activity towards *L. braziliensis* UA847, *L. tropica* EP41 and *L. major* Lm50122 with IC_{50} ranging from 0.8 to 13.7 μM, but not *L. amazonensis* LV78 and *L. major* FV1 (Table 1). They did not show toxicity towards macrophages RAW264.7 with IC_{50}>200 μM.

In series **B**, all hydroxyl groups in A ring have been removed from the flavonoid moieties. Flavonoid dimers with shorter linker length (compounds7 to **9** with 2 to 4 EG units) displayed no significant anti-promastigote activity. This result suggests that polar group in ring A is a favorable functional group for shorter flavonoid dimers. In contrast, flavonoid dimers with longer linker length (compounds **10** to **15** with 5 to 13av EG units) remain active towards CL promastigotes with IC_{50} ranging from 2.6 to 36 μM. Unfortunately, compounds **10**-**15** are toxic towards macrophages and therefore not useful.

In series **C**, hydrophobic substitutions at 3-, 6- or 7-position, such as methyl group (compounds **16**-**18**, **22**-**27**), ethyl group (compounds **19**-**21**) or fluoro group (compound **28**-**30**) were introduced into ring A or C. Compounds **20** and **26** showed potent anti-promastigote activity towards CL promastigotes (IC_{50} from 1.9 to 7.4 μM) with no toxicity towards macrophages.

(B) Amine-linked flavonoid dimers

Although flavonoid dimers **20** and **26** have significant anti-promastigote activity without toxicity toward macrophage RAW 264.7 cells, they are only sparingly soluble in aqueous medium possibly due to their hydrophobic property. Attempts to use them in animal experiments were impractical. We have previously synthesized a new class of flavonoid dimers with an amine group in the middle of the PEG linker (3). The amino group generally confers better aqueous solubility and thus better physicochemical properties for potential drug development. Compounds **31**-**50**, with different substituents on the amine nitrogen, were tested for their anti-promastigote activity (Table 2). Compound **31**, with R = H, was cytotoxic to both promastigotes and macrophage RAW 264.7 cells with IC_{50} value from 1.9 to 14.7 μM respectively. Comparing compound **31** with **9**, it is clear that the replacement of central oxygen by an amine in the linker changed the activity dramatically. Replacing the hydrogen with an ethyl group (compound **32**), hydroxyethyl group (compound **33**) or ethyl propanoate

group (compound **34**) on the amine group maintain cytotoxicity toward both promastigotes (IC_{50} for CL promastigotes = 1.3 to 11.0 μM) and RAW 264.7 cells (IC_{50} for RAW 264.7 = 6.4 to 16.0 μM).

Interestingly, when bulkier R groups were introduced to the amine nitrogen, the flavonoid dimers thus generated (compounds **35**-**44**) were generally non-toxic to RAW 264.7 cells with IC_{50} values ranging from 45.7 μM to greater than 100 μM (Table 2). For example, the *tert*-butyloxycarbonyl (Boc) group (compound **35**) displayed a marked anti-promastigote activity (IC_{50} = 2.3 to 4.7 μM) without toxicity towards RAW 264.7 cells. Introduction of a benzyl group (compound **36**) into the amino linker generated a compound containing no anti-promastigote activity and toxic effect to RAW 264.7 cells. When the benzyl group contained polar nitro group (compound **37**) or carboxylic ester group (compound **38**) at C-4 position, the anti-promastigote activity was also completely lost. In addition, the benzyl group containing a fluorine atom at C-2 (compound **45**), C-3 (compound **46**), C-4 (compound **47**) positions, two fluorine atoms at C-3 and C-4 (compound **48**) positions or three fluorine atoms at C-2, C-3 and C-4 positions (compound **49**) also resulted in complete loss of anti-promastigote activity. Nevertheless, a benzyl group with a trifluoromethyl group at C-4 position (compound **50**) improved anti-promastigote activity slightly when compared to compounds **45**-**49**. Placing the phenyl group further away from the nitrogen (compound **44**) and replacing the H atom with *N*-succinamide (compound **41**) and *N*-tosylate (compound **43**) resulted in low anti-promastigote activity Interestingly, replacing the *N*-tosylate with *N*-mesoylate (compound **42**) caused a marked anti-promastigote activity (IC_{50} for *L. braziliensis* UA847 and *L. tropica* EP41 = 0.4 to 0.8 μM).

*(C) Amine-linked **39**-derived flavonoid dimers*

Flavonoid dimers **39** and **40** are of particular interest. Introduction of a pyridine ring as part of R resulted in a very strong selective anti-promastigote activity. Compound **39** with nitrogen on position 4 of the pyridine ring has the highest anti-promastigote activity among all flavonoid dimers. No toxicity to the RAW 264.7 cells was observed. Compound **40**, containing nitrogen at position 2 of the pyridine ring, displayed about at least 27-fold lower anti-promastigote activity than compound **39**, indicating that the position of nitrogen atom on the pyridine ring is of critical importance for anti-promastigote activity.

It was found that compound **39** which possesses a pyridine ring at the amine linker showed the most potent anti-promastigote and anti-amastigote activity towards visceral *L. donovani* (24). Here, compound **39**also displayed significant anti-promastigote activity towards all strains of CL promastigotes with IC_{50} from 0.19 to 0.69 μM (Table 2). The position of the nitrogen atom in the pyridine ring is important in determining the anti-promastigote activity. The rank order of anti-promastigote activity was as follows: *para*-position (compound **39**; IC_{50} ranged 0.19 to 0.69 μM) >*meta*-position (compound **68**; IC_{50} ranged from 0.5 to 8.0 μM) >>*ortho*-position (compound **40**; IC_{50} ranged from 5.1 to > 100 μM). In general, a bromo substitution at *meta*-position (compound **69**) or *ortho*-position (compound **71**) and a cyano group at *meta*-position (compound **70**) of the pyridine ring reduced or completely destroy the anti-promastigote activity. Moreover, replacement of pyridine ring by pyrimidine ring (compound **72**) exhibited at least 2-fold lower anti-promastigote activity as compared to the parent compound **39**. Finally, compound **73** completely lost the anti-promastigote activity probably due to bulkiness of quinidine ring.

Linker length and attachment position of the two flavones to the amino PEG linker are also important factors in determining the antileishmanial activity. Compound **53** (with shorter amino PEG linker than compound 39) has lost the anti-promastigote activity. Attachment at C-3' position of B-ring (compound **56**) did not change the anti-promastigote activity significantly compared to compound **39** which has linker attached at C-4' position. However, attachment at C-2' position of B-ring (compound **57**) or C-3 position of C-ring (compound **58**) resulted in at least 10-fold reduction in anti-promastigote activity as compared to parent compound **39**. C3'-methoxy substitution in the B-ring (compound **59**) caused a remarkable cytotoxicity towards both promastigotes and host PEM cells. On the contrary, C3-methoxy substitution in the C-ring (compound **60**) or addition of a fluorine atom at C-6 position of A-ring (compound **61**) did not cause any toxic effect.

*(D) Comparison of anti-promastigote activity of **39** with other antileishmanial agents.*

We compared compound **39** with other antileishmanials namely pentamidine, amphotericin B and miltefosine (Table 3). Second-line antileishmanials like pentamidine and miltefosine displayed moderate anti-promastigote activity (IC_{50} values ranging from 1.1 to 17.8 μM and 5.3 to 32.7 μM, respectively) and moderate cytotoxicity towards PEM cells (IC_{50} values of 30.4 μM and 75.3 μM, respectively). Amphotericin B has the highest anti-promastigote activity (IC_{50} values ranging from 0.13 to 0.34 μM). However, it was also the

most toxic compound for PEM cells (IC_{50} of 7.4 μM) (Table 3). In comparison, compound **39** was as potent as amphotericin B in killing promastigotes with IC_{50} values ranging from 0.19 to 0.69 μM (Table 3). Importantly, compound **39** was not toxic to PEM cells (IC_{50} >88 μM respectively) (Table 3).

We have also determined the therapeutic index of these antileishmanial agents, defined as the ratio of IC_{50} against PEM cells to IC_{50} against-promastigotes. A therapeutic index less than 10 would indicate probable non-selective cytotoxicity for the tested compounds (22). As shown in Table 3, compound **39** has the highest therapeutic index (from >127.5 to >463.2), followed by amphotericin B (21.1 to 56.9). Other compounds displayed a significantly lower therapeutic index ranging from 1.7 to 27.6.

***In vitro* anti-amastigote activity of flavonoid dimer 39 and its derivatives**. Here, we investigated if compound **39** has anti-amastigote activity against *L. amazonensis* LV78 and *L. braziliensis* UA847. Intracellular amastigotes were obtained by infecting PEM cells with late log-phase *L. amazonensis* LV78 and *L. braziliensis* UA847 promastigotes. A very pronounced anti-amastigote activity was noted for compound **39** compared to solvent control. Numerous amastigotes were observed in host macrophages in control (Fig. 1A and 1D) whereas only a few amastigotes or none were noted in the compound **39**-treated *L. amazonensis* LV78 (2 μM, Fig. 1B) and *L. braziliensis* UA847 (2 μM, Fig. 1E). Percentage of macrophage infected was decreased from 81.0% in solvent control to 2.0% in compound **39**-treated *L. amazonensis* LV78 (Fig. 1C) and from 51.0% to 0.6% in **39**-treated *L. braziliensis* UA847 (Fig. 1F). Number of amastigotes per 100 macrophages was also reduced from 324 to 3 (Fig. 1C) and from 99 to 1 (Fig. 1F) after adding 2 μM of compound **39**. These results suggest that compound **39** has a potent anti-amastigote activity.

We have compared compound **39** and its derivatives (compounds **39**, **40**, **42**, **60**, **61**, **68** and **72**) with amphotericin B and SSG in terms of their anti-amastigote activity and therapeutic index. All flavonoid dimers (except compound **61**) showed promising anti-amastigote activity towards *L. amazonensis* and *L. braziliensis* with IC_{50} below 10 μM. Compound **39** was the most potent with IC_{50} below 2.2 μM (Table 4). Same as anti-promastigote activity, position of nitrogen atom in the pyridine is important in determining the anti-amastigote activity. Compound with nitrogen atom at *para*-position (compound **39**) has stronger leishmanicidal activity than that with nitrogen atom at *meta*-position (compound **68**) and *ortho*-position (compound **40**). Addition of a fluorine atom at C-6 position of A-ring

(compound **61**) and replacing the pyridine by pyrimidine (compound **72**) resulted in reduction of anti-amastigote activity. Amphotericin B has the highest anti-amastigote activity (0.049 to 0.360 μM) followed by compound **39** (0.17 to 2.2 μM). Compound **42** with mesoylate group at the amine linker exhibited moderate anti-amastigote activity (IC_{50} = 6.5 to 9.0 μM towards *L. amazonensis* and *L. braziliensis)* (Table 4). SSG exhibited the weakest anti-amastigote activity with IC_{50} ranged from 18.5 to 42.0 μM. Amphotericin B, however, was also the most toxic compound towards PEM cells (IC_{50} = 7.4 μM). In contrast, compound **39**, was almost non-toxic to PEM cells (IC_{50}> 88 μM). Putting together, the therapeutic index of compound **39** (therapeutic index ranged from >40.0 to >517.6) was slightly higher than that of amphotericin B (therapeutic index ranged from 20.6 to 151.0), suggesting that both of them possess very high selective cytotoxicity for *Leishmania* amastigotes.

***In vivo* antileishmanial activity of flavonoid dimer 39.** Compound **39** was tested for its antileishmanial efficacy in a murine model of cutaneous leishmaniasis: Balb/c mice infected with *L. amazonensis* LV78 in the footpad. Measurement of lesion thickness was used as an indicator of disease progression. Intralesional administration of hydrochloride salt of compound **39** (**39.HCl**) (2.5 mg/kg [3 mM] every 4 days for 8 times) can inhibit the growth of lesion as efficiently as SSG (28 mg/kg [31 mM] every 4 days for 8 times) (Fig. 2A). On 48 days post-infection, lesion thickness of footpad in **39.HCl-** and SSG-treated mice were reduced by 32% (P<0.05) and 36% (P<0.05) of PEG control, respectively (Fig. 2B) whereas footpad weight was reduced to 67% (P<0.05) and 47% (P<0.005) of PEG control, respectively (Fig. 2C). Parasite burden of the footpad in **39.HCl**- and SSG-treated mice, as measured by the number of amastigotes recovered from infected footpad after *in vitro* culture, were reduced to 6% (P<0.001) and 5% (P<0.001) of PEG control, respectively (Fig. 2D). Efficacy of **39.HCl** was dose-dependent because only 2.5 mg/kg of **39.HCl** was effective in reducing lesion thickness/weight or parasite burden, but not 0.5 mg/kg (Fig. 2B and 2C). Throughout the whole period of experiment, there was no significant difference in the body weight of mice from different groups (data not shown), suggesting that neither **39.HCl** nor SSG exhibit any significant toxicity to the animals at the indicated dosage.

In summary, we found that flavonoid dimer **39** was potent against both cutaneous promastigotes (IC_{50} = 0.19-0.69 μM) and amastigotes (IC_{50} = 0.17-2.2 μM). Its activity was comparable to or slightly lower than that of amphotericin B (IC_{50} = 0.13-0.34 μM for promastigotes and 0.049-0.360 μM for amastigotes) and better than SSG (Table 3 and 4).

39.HCl at 2.5 mg/kg every 4 days for 8 times can reduce the parasite burden in a mouse model of CL by 20-fold to 5-6% of control. Together with its low toxicity against macrophages, **39** is a potential candidate compound for further development into antileishmanial drug for treating cutaneous leishmaniasis.

DISCUSSION

Medicinal plants against parasitic disease have been studied for a long time (12). Flavonoids, abundantly present in fruits, vegetables and tea, can provide a good source of compounds for designing antileishmanial drugs (17, 21). Two important members of the flavonoid family, namely quercetin and luteolin, have been reported to have marked antileishmanial activity (7, 9, 15, 18). Based on monomeric flavonoids, we have previously synthesized a library of flavonoid dimers and demonstrated that they represent a new class of potent and safe antileishmanial agents against *L. donovani* promastigotes and amastigotes (24). Of particular interest is compound **39** which contains a pyridine ring at amine PEG linker. Not only it was more potent than monomeric quercetin, luteolin and other bioflavonoids, its activity is comparable to that of amphotericin B (24).

Here, by screening the flavonoid dimer library we previously synthesized, we found that flavonoid dimer is active against different strains of CL-causing *Leishmania* species, including *L. amazonensis*, *L. braziliensis*, *L. tropica* and *L. major*. Among the flavonoid dimers screened, compound **39** has the highest anti-promastigote activity (IC_{50} = 0.19-0.69 μM). Although the anti-amastigote activity of compound **39** (IC_{50} = 0.17-2.2 μM) was lower than that of amphotericin B (IC_{50} = 0.05-0.36 μM), its therapeutic index (>40 to >518) was higher than that of amphotericin B (21 to 151) due to its low toxicity towards macrophages (IC_{50}> 88 μM) (Table 4).

The salt form of compound **39**, **39.HCl**, was as potent as SSG in reducing the lesion thickness/weight in a cutaneous leishmaniasis animal model, even when 10-fold less of **39.HCl** was used. When used at 2.5 mg/kg, one intralesional injection for every 4 days for 8 times, **39.HCl** can reduce the parasite burden in the infected footpad to 6% of that of the solvent control (Fig. 2D). Reduction of dose to 0.5 mg/kg abolished the activity of **39.HCl** (Fig. 2D). We did not observe any obvious toxicity symptoms like death or significant weight loss in the **39.HCl**-treated animals. Compared to SSG which has many drawbacks including

toxic side effect, need for intramuscular administration and emergence of drug resistance (2), **39.HCl** seems to be safer to use.

One potential drawback of **39** is relatively low solubility in aqueous solutions. Introduction of an amine group into flavonoid dimers, together with the use of the salt form (**39.HCl**) instead of free base (**39**), did increase the water solubility. Despite these, **39.HCl** still has relatively low water solubility and requires the use 50% PEG as solvent in animal studies. Formulation studies are ongoing to search for better solvents. Future studies should include the trial of different administration routes in animal studies (topical, intravenous, intraperitoneal or oral approach). A pharmacokinetics profile and extensive toxicity study will also be necessary. Here, intralesional administration of **39.HCl** at the dosage applied did not show any observable signs of toxicity in mice.

It has been reported that quercetin can induce the death of *L. amazonensis* by increasing the production of reaction oxygen species and collapsing the mitochondrial potential (9). Quercetin has also been reported to have multiple targets including arginase which is an important enzyme in polyamine biosynthesis pathway (7), topoisomerase II in kinetoplast which induces DNA cleavage leading to apoptosis (15) and iron metal which is important for growth and replication of parasite (18). The mechanism of action and molecular targets of **39** remain to be studied.

In summary, we have discovered that flavonoid dimers, particularly compound **39** and its salt form **39.HCl**, are effective against different strains of CL-causing *Leishmania* species in both *in vitro* and *in vivo* studies. Together with its low toxicity towards macrophages, we hope flavonoid dimers can be further developed in the future to be used clinically against CL.

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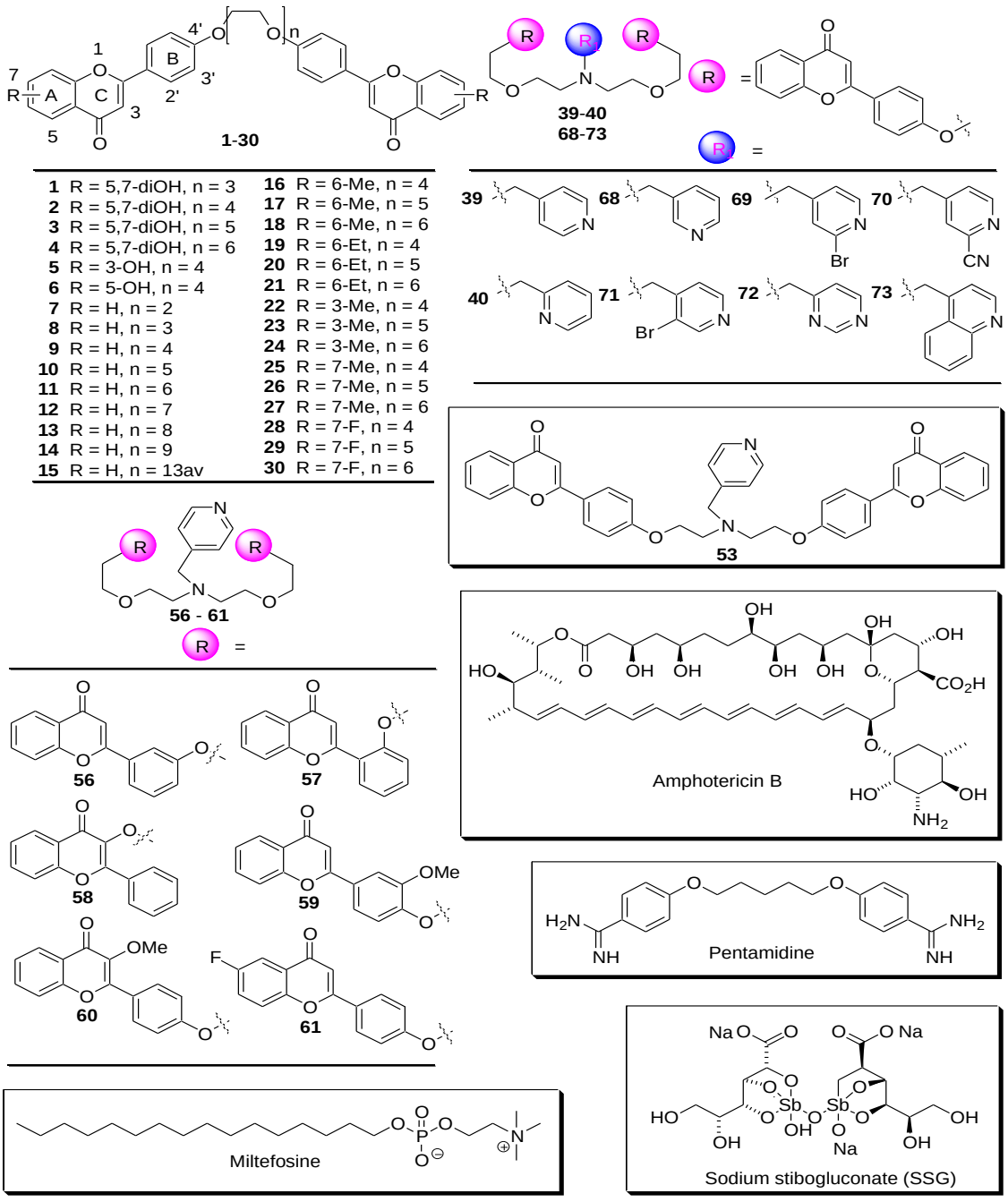
FIGURE LENEND

Chart 1 Chemical structures of previously reported flavonoid dimers **1** – **73**[a] and other antileishmanial compounds used in this study



[a]PEG 600 with average number of EG unit of 13 was used to synthesize compound **15**.

TABLE 1 Anti-promastigote activity of synthetic flavonoid dimers with PEG linkers

		IC_{50} (μM)					
		CL promastigotes					Macrophages
		L. amazonensis	*L. braziliensis*	*L. tropica*	*L. major*	*L. major*	RAW264.7[b]
Cpds[a]	Series	LV78	UA847	EP41	FV1	Lm50122	
1		>100	2.4 ± 1.6	3.0 ± 1.6	>100	0.8 ± 0.4	>200
2		>100	5.0 ± 2.0	13.7 ± 8.6	>100	0.9 ± 0.0	>200
3	A	>100	>100	>100	>100	ND	>200
5		>100	>100	>100	>100	ND	ND
6		>100	>100	>100	>100	ND	ND
7		>100	>100	>100	>100	ND	>100
8		>100	>100	>100	>100	ND	>95
9		>100	>100	>100	>100	ND	>100
10		12.1 ± 4.9	5.2 ± 1.6	11.4 ± 4.3	29.5	ND	8.9 ± 2.3
11	B	6.2 ± 1.8	2.6 ± 1.0	3.9 ± 0.4	9.5	ND	7.1 ± 1.2
12		4.9 ± 0.9	3.6 ± 0.6	4.9 ± 0.3	7.1	ND	4.8 ± 0.3
13		6.2 ± 1.7	3.3 ± 0.4	4.5 ± 0.5	7.9	ND	4.4 ± 0.4
14		5.1 ± 0.6	5.6 ± 1.9	5.7 ± 0.4	7.1	ND	8.1 ± 1.2
15		22.6 ± 4.3	31.0 ± 8.9	35.5 ± 9.1	29.4	ND	8.4 ± 2.4
16		>100	>100	>100	>100	ND	>100
17		>100	>100	>100	>100	ND	>100
18		>100	>100	13.3 ± 4.4	>100	ND	>100
19		>100	>100	>100	>100	ND	>100
20		7.4 ± 3.7	3.3 ± 1.3	1.9 ± 0.2	>100	3.0 ± 0.1	>200
21		9.4 ± 1.9	2.9 ± 1.1	2.3 ± 0.2	11.1	ND	5.5 ± 0.7
22		9.4 ± 3.3	6.8 ± 4.8	3.1 ± 0.1	>100	ND	8.1 ± 0.6
23	C	7.9 ± 3.7	1.6 ± 0.5	1.7 ± 0.2	6.0	ND	3.1 ± 1.3
24		2.0 ± 0.2	1.8 ± 0.7	1.7 ± 0.1	3.9	ND	2.3 ± 0.4
25		>100	2.8 ± 1.5	>100	>100	ND	88.6 ± 4.5
26		4.2 ± 1.2	2.1 ± 0.5	3.1 ± 0.4	6.2	2.4 ± 0.2	>80
27		2.9 ± 0.6	2.1 ± 0.7	1.9 ± 0.2	3.9	ND	5.3 ± 2.1
28		41.3 ± 13.7	12.4 ± 3.8	22.8 ± 5.9	>100	1.0 ± 0.0	>200
29		>100	4.0 ± 0.5	>100	>100	5.6 ± 2.0	>200
30		6.1 ± 1.7	2.8 ± 0.2	4.6 ± 1.2	16.8	ND	7.8 ± 1.1

Synthetic flavonoid dimers were grouped into three series (A to C) with various linker lengths containing different EG units (n). Substitutions were made at A-ring of flavone. IC_{50} values for anti-promastigote activity towards *L. amazonensis* LV78, *L. braziliensis* UA847, *L.*

tropica EP41 and *L. major* FV1and *L. major* Lm50122 were shown. The values were presented as mean ± standard error of mean. N = 1-4 independent experiments. ND = not determined. [a]All compounds were dissolved in DMSO and the highest % of DMSO used was 1% at which no toxicity to promastigotes and RAW 264.7 cells was observed. [b]The data of compound toxicity towards macrophage RAW 264.7 cell line has been published (24).

TABLE 2 Anti-promastigote activities of amine-linked and **39**-derived flavonoid dimers

	IC_{50} (μM)						
	CL Promastigotes					Macrophages[b]	
	L. amazonensis	*L. braziliensis*	*L. tropica*	*L. major*	*L. major*		
Cpds[a]	LV78	UA847	EP41	FV1	Lm50122	RAW264.7	PEM
31	14.7 ± 5.5	1.9 ± 0.3	9.1 ± 3.1	ND	ND	2.1 ± 0.9	ND
32	8.1 ± 1.6	1.3 ± 0.1	1.9 ± 0.6	ND	ND	8.3 ± 1.0	ND
33	5.9 ± 1.1	2.5 ± 1.1	3.2 ± 1.7	ND	ND	6.4 ± 1.5	ND
34	11.0 ± 2.0	2.4 ± 0.4	5.2 ± 1.6	ND	ND	16.0 ± 6.2	ND
35	4.4 ± 1.0	2.3 ± 0.2	4.7 ± 2.3	ND	ND	>100	ND
36	>100	>100	99.3	ND	ND	>79	ND
37	>100	>100	40.0 ± 2.4	ND	>100	70.3 ± 9.7	ND
38	>100	>100	>57	ND	>100	45.7 ± 19.1	ND
39	0.43 ± 0.09	0.43 ± 0.11	0.19 ± 0.03	0.69 ± 0.04	0.38 ± 0.03	>100	>88
40	39.2 ± 11.8	33.5 ± 14.8	5.1 ± 0.9	>100	21.8 ± 6.3	53.0 ± 12.7	22.7 ± 4.7
41	>100	>100	>88	ND	ND	65.0	ND
42	>100	0.8 ± 0.3	0.4 ± 0.0	ND	ND	96.0 ± 4.0	ND
43	>100	>100	>100	ND	ND	>100	ND
44	>100	48.2 ± 0.2	>79	ND	ND	85.0	ND
45	>100	>100	>100	ND	ND	ND	ND
46	>100	>100	>100	ND	ND	ND	ND
47	>100	>100	>100	ND	ND	ND	ND
48	>100	>100	>100	ND	ND	ND	ND
49	>100	>100	>100	ND	ND	ND	ND
50	35.3 ± 14.2	9.0 ± 3.2	12.9 ± 7.8	ND	ND	ND	ND
53	>100	>100	0.8 ± 0.1	>100	ND	ND	>58
56	1.5 ± 0.8	0.8 ± 0.1	0.3 ± 0.0	1.6 ± 0.3	3.4 ± 1.8	ND	>50
57	ND	ND	ND	ND	5.3 ± 1.8	ND	>11
58	4.3	4.3	4.5	29.1	7.4 ± 4.9	ND	>33
59	1.5 ± 0.2	1.4 ± 0.3	0.6 ± 0.1	1.7 ± 0.6	2.4 ± 0.9	ND	2.7 ± 0.3
60	1.8 ± 0.4	1.6 ± 0.2	0.7 ± 0.1	3.0 ± 0.2	4.6 ± 2.3	ND	>50
61	0.6 ± 0.1	0.7 ± 0.1	0.3 ± 0.0	0.8 ± 0.2	3.1 ± 0.2	ND	>100
68	7.7 ± 1.8	6.3 ± 1.1	0.5 ± 0.1	5.6 ± 2.8	8.0	ND	33.1 ± 18.9
69	>100	>100	1.0 ± 0.3	>100	>50	ND	>100
70	>100	>100	33.3 ± 12.5	>100	>50	ND	>92
71	2.4 ± 0.7	>100	0.4 ± 0.0	>100	>50	ND	>100
72	3.2 ± 1.2	1.7 ± 0.5	0.4 ± 0.1	3.2 ± 0.4	4.3 ± 1.6	ND	>33
73	>100	>100	7.7 ± 3.9	>100	ND	ND	ND

Flavonoid dimers containing amino PEG linker (compounds **31-50**, **53**, **56-61** and **68-73**) with different substitutions on the linker were synthesized and tested for their cytotoxicity towards different cutaneous promastigotes, RAW264.7 and PEM cells. IC_{50} values were presented as mean ± standard error of mean. N = 1-4 independent experiments. ND = not

481 determined. [a]All compounds were dissolved in DMSO and the highest % of DMSO used was
482 1% at which no toxicity to promastigotes, RAW264.7 and PEM was observed. [b]The data of
483 compound toxicity towards macrophage RAW264.7 and PEM cells has been published (24).

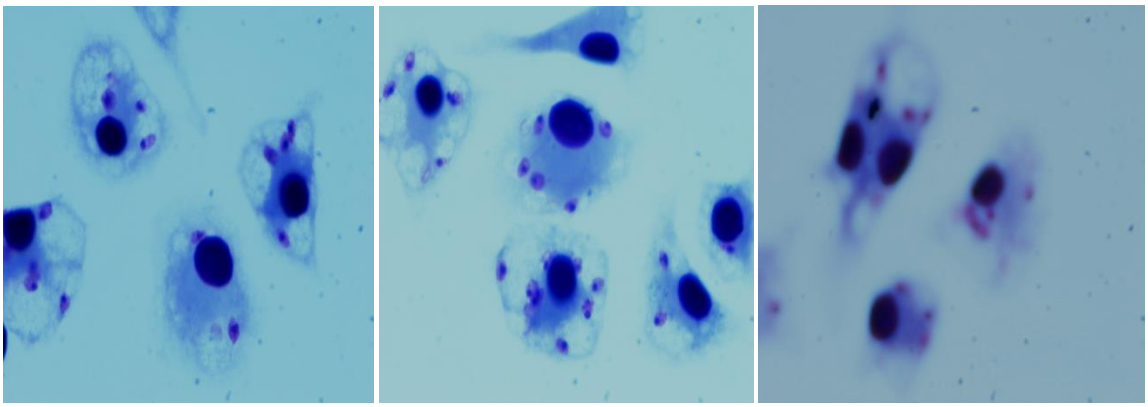
TABLE 3 Anti-promastigote activities and therapeutic index of standard antileishmanials compared to **39**

	Promastigotes										Macrophages
	L. amazonensis LV78		*L. braziliensis* UA847		*L. tropica* EP41		*L. major* FV1		*L. major* Lm50122		PEM
Cpds	IC_{50} (µM)	Therapeutic index	IC_{50} (µM)	Therapeutic index	IC_{50} (µM)	Therapeutic index	IC_{50} (µM)	Therapeutic index	IC_{50} (µM)	Therapeutic index	IC_{50} (µM) [c]
Pentamidine[a]	15.7 ± 5.2	1.9	1.1 ± 0.1	27.6	14.1 ± 5.4	2.2	17.8 ± 2.2	1.7	7.7 ± 1.0	3.9	30.4 ± 10.5
Miltefosine[a]	32.7 ± 26.9	2.3	13.0 ± 1.3	5.8	5.3 ± 1.0	14.2	9.7 ± 1.1	7.8	9.8 ± 2.3	7.7	75.3 ± 9.4
Amphotericin[b]	0.24 ± 0.03	30.8	0.17 ± 0.03	43.5	0.13 ± 0.01	56.9	0.29 ± 0.05	25.5	0.34 ± 0.06	21.1	7.4 ± 0.4
39[b]	0.43 ± 0.09	>204.7	0.43 ± 0.11	>204.7	0.19 ± 0.03	>463.2	0.69 ± 0.04	>127.5	0.38 ± 0.03	>231.6	>88

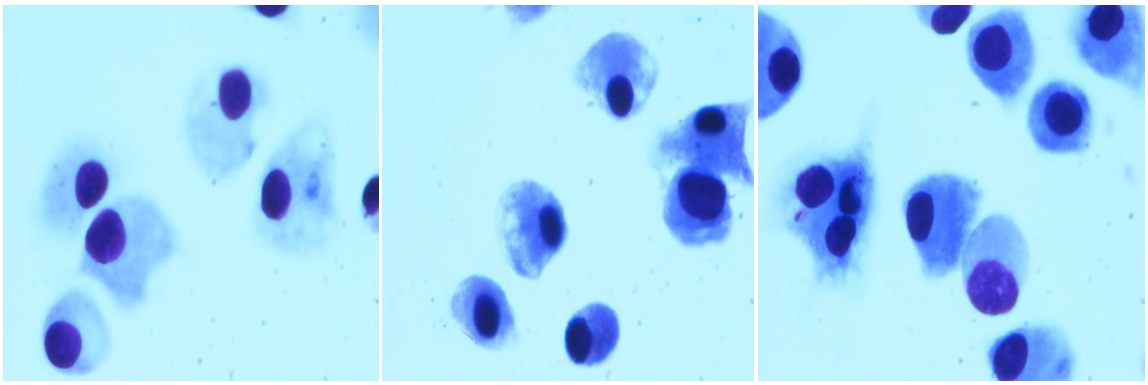
IC_{50} values of current antileishmanials and flavonoid dimer **39** towards promastigotes (*L. amazonensis* LV78, *L. braziliensis* UA847, *L. tropica* EP41, *L. major* FV1and *L. major* Lm50122) and macrophages (PEM) were determined. IC_{50} values were presented as mean ± standard error of mean. N = 2-4 independent experiments. Therapeutic index was defined as the ratio of IC_{50} of antileishmanials towards PEM over promastigotes. [a] Compounds were dissolved in sterile H_2O. [b] Compounds were dissolved in DMSO. No toxicity to the PEM cells was observed at 1% DMSO. [c] The data of compound toxicity towards macrophage PEM has been published (24).

FIG 1 Anti-amastigote activity of compound **39** against *L. amazonensis* LV78 and *L. braziliensis* UA847 amastigotes grown in PEM cells

A) 0.1% DMSO control (*L. amazonensis* LV78, 1000x magnification)



B) 2 µM **39** (*L. amazonensis* LV78, 1000x magnification)



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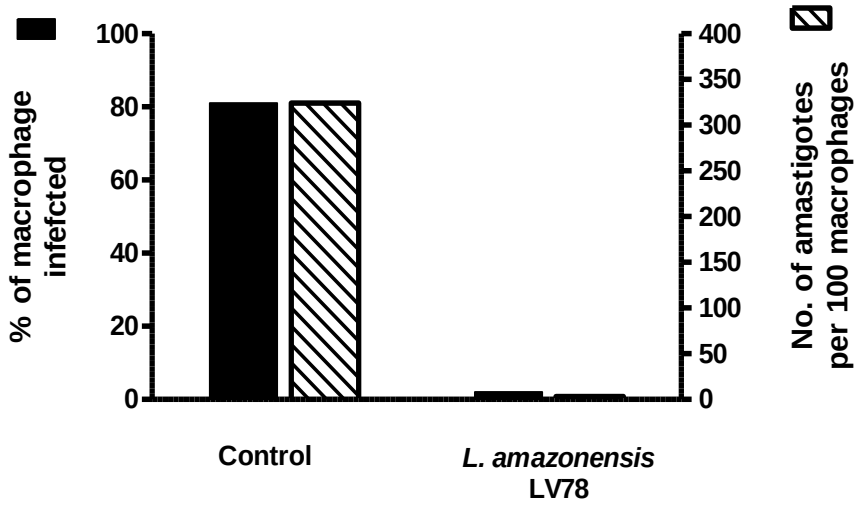
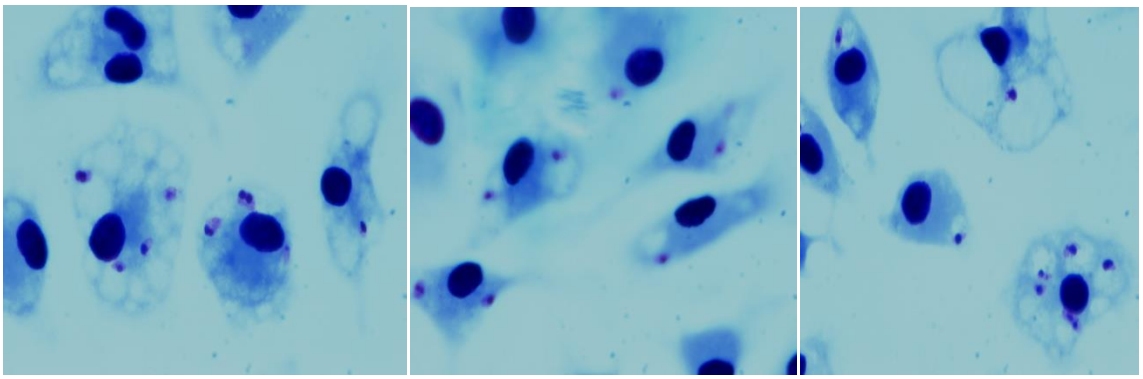
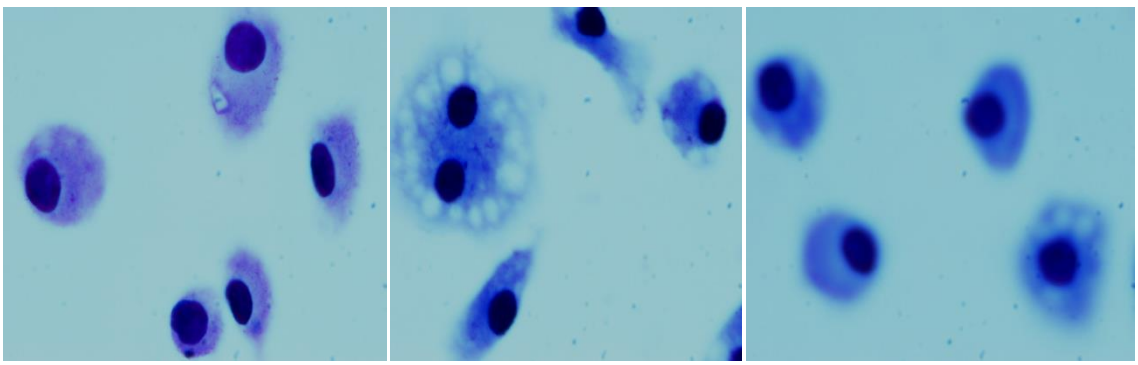


D) 0.1% DMSO (*L. braziliensis* UA847, 1000x magnification)



E) 2 µM **39** (*L. braziliensis* UA847, 1000x magnification)



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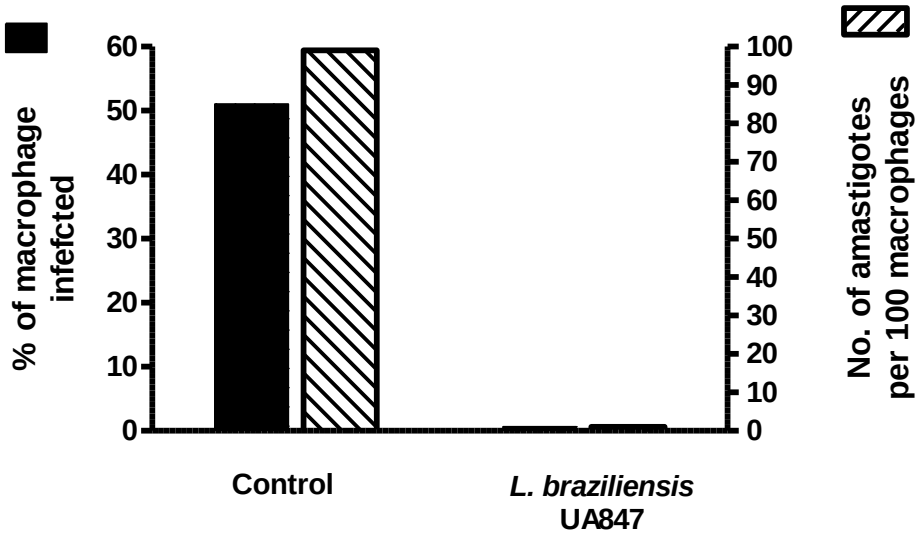


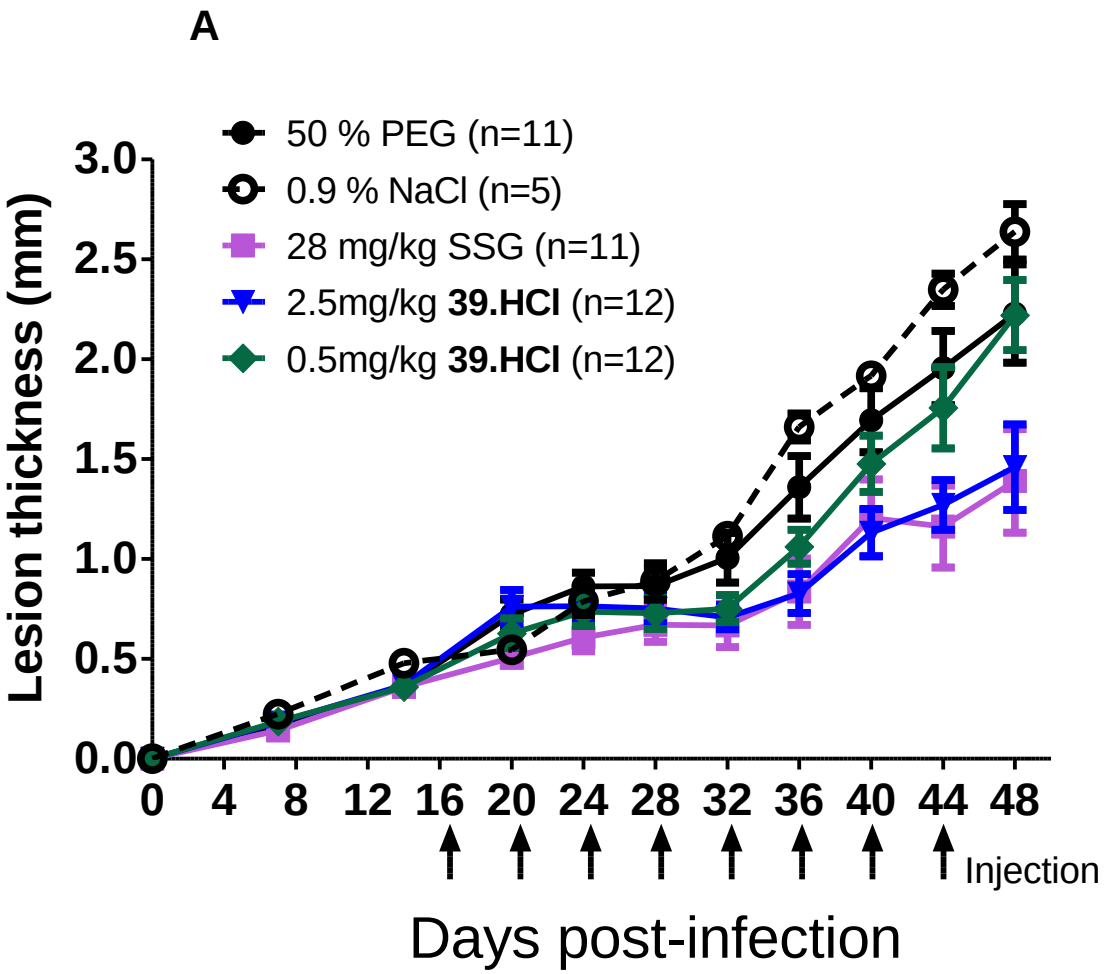
Anti-amastigote activity of compound **39** against cutaneous *L. amazonensis* LV78 and *L. braziliensis* UA847 grown in PEM cells. PEM cells were infected with log stage promastigotes for 24 hours at 37^{o}C. Infected macrophages were then treated with either 0.1% DMSO or 2 µM compound 39 for 3 days at 37^{o}C. (A) *L. amazonensis* LV78 with 0.1% DMSO (B) *L. amazonensis* LV78with 2 µM of **39**, (D) *L. braziliensis* UA847 with 0.1% DMSO and (E) *L. braziliensis* UA847 with 2 µM of **39**. Three representative microscopy pictures are shown in each treatment group. After 3 days, the cover slips were stained with Giemsa. Percentage of macrophage infected and the number of amastigotes per 100 macrophages were determined (C) *L. amazonensis* LV78 and (F) *L. braziliensis* UA847. The black column represents the percentage of macrophage infected and the stripped column represents the number of amastigotes per 100 macrophages.

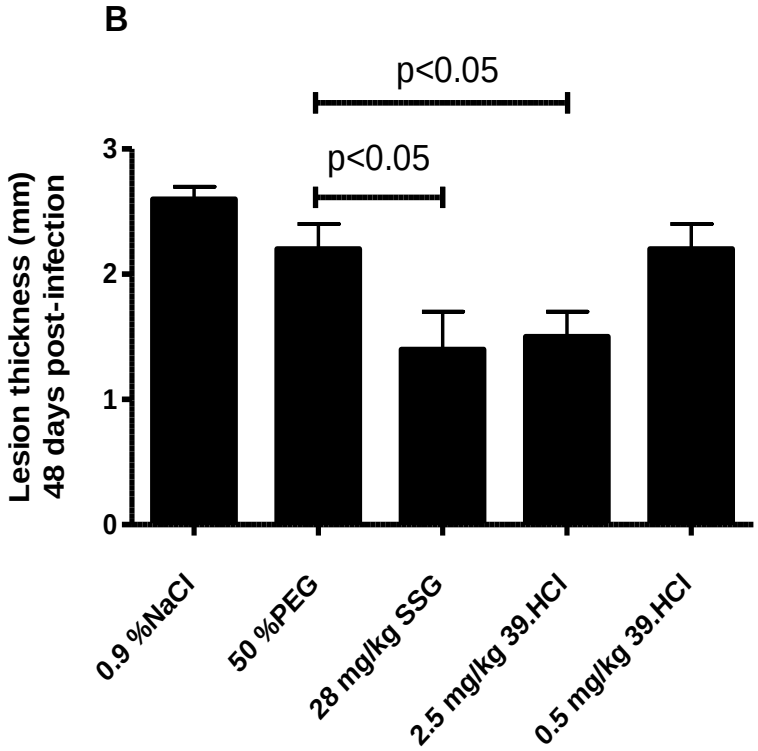
TABLE 4 Anti-amastigote activity and therapeutic index of SSG and amphotericin B compared to **39** and its derivatives

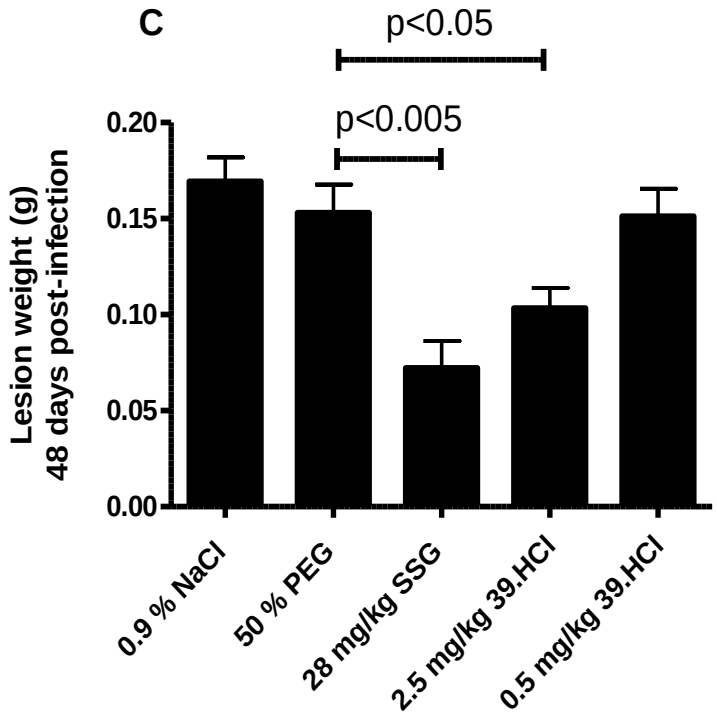
	Amastigotes								Macrophages	
	L. amazonensis LV78		*L. braziliensis* UA847		*L. tropica* EP41		*L. major* FV1		RAW264.7	PEM
Cpds	IC_{50} (μM)	Therapeutic index	IC_{50} (μM)	Therapeutic index	IC_{50} (μM)	Therapeutic index	IC_{50} (μM)	Therapeutic index	IC_{50} (μM)	IC_{50} (μM)
Amphotericin B	0.055 ± 0.029	134.5	0.049 ± 0.011	151.0	0.076 ± 0.023	97.4	0.360 ± 0.06	20.6	ND	7.4 ± 0.4
SSG	32.5 ± 17.6	>338.5	35.6 ± 19.9	>308.9	18.5 ± 0.5	>594.6	42.0	>261.9	ND	>11000
39	0.37 ± 0.07	>237.8[a]	0.17 ± 0.02	>517.6[a]	1.8 ± 0.7	>48.9[a]	2.2 ± 1.1	>40.0[a]	>100	>88
40	1.1 ± 0.5	20.6[a]	4.4 ± 1.7	5.2[a]	>10	<2.3[a]	ND	ND	53.0 ± 12.7	22.7 ± 4.7
42	6.5 ± 1.0	14.8[b]	9.0	10.7[b]	ND	ND	ND	ND	96.0 ± 4.0	ND
60	2.1 ± 0.4	>23.8	3.3 ± 1.1	>15.2	>10	<5.0	ND	ND	ND	>50
61	>10	<10.0	>10	<10.0	>10	<10.0	ND	ND	ND	>100
68	1.2 ± 0.1	27.6	0.90	36.8	>10	<3.3	ND	ND	ND	33.1 ± 18.9
72	2.9 ± 0.4	>11.4	5.0 ± 1.8	>6.6	>10	<3.3	ND	ND	ND	>33

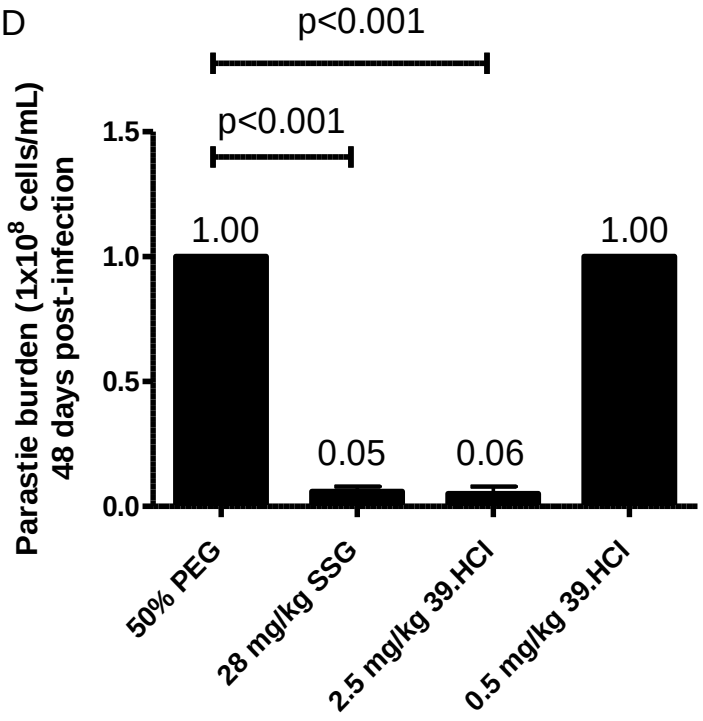
PEM cells were infected with log stage of *L. amazonensis* LV78, *L. braziliensis* UA847, *L. tropica* EP41, *L. major* FV1for 24 hours at 37°C. Infected macrophages were then treated with various antileishmanials and incubated for 3 days at 37°C. After 3 days, the cover slips were stained with Giemsa. The number of amastigotes per 100 macrophages was determined and used to calculate IC_{50} values. The values were presented as mean ± standard error of mean. N = 1-3 independent experiments. Therapeutic index was determined by dividing IC_{50} towards RAW264.7 or PEM cells over IC_{50} towards cutaneous amastigotes. [a] Therapeutic indexe s are normalized to the PEM cells. [b]Therapeutic indexes are normalized to the RAW264.7 cells. ND = not determined.

FIG 2 *In vivo* antileishmanial efficacy of flavonoid dimer **39.HCl**




B
p<0.05
p<0.05
Lesion thickness (mm)
48 days post-infection
3
2
1
0
0.9 %NaCl
50 %PEG
28 mg/kg SSG
2.5 mg/kg 39.HCl
0.5 mg/kg 39.HCl


C
p<0.05
p<0.005
Lesion weight (g)
48 days post-infection
0.20
0.15
0.10
0.05
0.00
0.9 % NaCl
50 % PEG
28 mg/kg SSG
2.5 mg/kg 39.HCl
0.5 mg/kg 39.HCl



Balb/c mice were infected in the footpad by subcutaneous infection with $1x10^7$ log-phase promastigotes of cutaneous *L. amazonensis* LV78. The treatment included 0.9% NaCl, 50% PEG, 28 mg/kg SSG, 2.5 mg/kg **39.HCl** and 0.5 mg/kg **39.HCl**. (A) Growth rate of lesion. The drugs were intralesionally injected every 4 days for 8 times, starting from 16 days post-infection. The lesion thickness was plotted as an indicator of disease progression. (B) Lesion thickness on 48 days post-infection. (C) Lesion weight on days 48 days post-infection. (D) Parasite burden of mice on 48 days post-infection. *P*-values between the solvent group (50% PEG) and treatment groups (28 mg/kg SSG, 2.5 mg/kg **39.HCl** or 0.5 mg/kg **39.HCl**) were calculated using Student t's test for two paired samples. The experimental value was considered as significant when the *p*-value was smaller than 0.05. The values were presented as mean ± standard error of mean.