Structure and antiparasitic activity relationship of alkylphosphocholine analogues against *Leishmania donovani.*

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Abstract

Miltefosine (Milt) is the only oral treatment for visceral leishmaniasis (VL) but its use is associated with adverse effects e.g. teratogenicity, vomiting, diarrhoea. Understanding how its chemical structure induces cytotoxicity, whilst not compromising its anti-parasitic efficacy, could identify more effective compounds. Therefore we systemically modified the compound's head, tail and linker tested the *in vitro* activity of three alkylphosphocholines (APC) series against *Leishmania donovani* strains with different sensitivities to antimony. The analogue, APC12, with an alkyl carbon chain of 12 atoms, was also tested for anti-leishmanial *in vivo* activity in a murine VL model. All APCs produced had anti-leishmanial activity in the micromolar range (IC_{50} and IC_{90}, 0.46 μM - >82.21 μM and 4.14 μM - 739.89 μM; 0.01 - >8.02 μM and 0.09 μM - 72.18 μM respectively against promastigotes and intracellular amastigotes). The analogue, APC12 was the most active, was 4-10 fold more effective than the parent Milt molecule (APC16), irrespective of the strain's sensitivity to antimony. Intravenous administration of 40 mg/kg APC12 to *L. donovani* infected BALB/c mice reduced liver and spleen parasite burdens by 60 ± 11% and 60 ± 19% respectively while oral administration reduced parasite load in the bone marrow by 54 ± 34%. These studies confirm that it is possible to alter the Milt structure and produce more active anti-leishmanial compounds.

Key words: *Leishmania donovani*, miltefosine, SAR

Introduction

Visceral leishmaniasis (VL) is a devastating disease for millions of people, primarily in East Africa, South Asia, South America, and Mediterranean Region. The World Organisation estimates that there are 200,000-400,000 people at risk of infection and that the disease is responsible for approximately 20,000-40,000 deaths/year. Despite a campaign to eliminate the disease by 2020 there are still VL cases in endemic countries such as Brazil [1]. Currently there is no clinical vaccine for the disease therefore control is reliant on preventive measures on preventing people from getting infected, vector control and treatment of active cases. There are a limited number of drugs that can be used for the treatment of VL and some have been discontinued due to a reduction in clinical efficacy caused by non-compliance with treatment regimens and the emergence of drug resistant parasites in endemic areas [2-4]. The only oral drug for VL is the alkylphosphocholine (APC), miltefosine (Milt), a repurposed anti-cancer drug, which is the recommended second-line treatment for VL in India. Although Milt is a highly effective drug, its use is associated with a high relapse rate due to factors such as non-compliance by patients to the recommended treatment regimen, the drug's long half-live which helps select for drug resistant parasites [5], and naturally occurring Milt resistant parasites [6]. For example, VL monotherapy with Milt was associated with a 20% relapse rate in Nepal at 12 months post-treatment [7]. Despite this drawback Milt may have a role in combating the emergence of drug resistance as part of a combination treatment. It has recently been used in combination with paromomycin (PMM) or amphotericin B (AMB) to determine which regimen was most effective in curing VL and preventing relapse [8]. At 12 months post-treatment the cure rate for treatment with AMB alone was 93.7%, AMB/Milt was 91.5% and Milt/PMM 98.6%. It is possible that changing the structure of Milt parent compound may allow the development of a drug that is still as active, has a shorter half live and is less toxic. Studies have shown that APCs with short alkyl carbon chain do have shorter half-life [9], and alterations on the hydrophilic choline head, the alkyl carbon tail and linker of this molecule have produced compounds with differential efficacy and pharmacokinetics [10-12].

Therefore, in this study we produced APCs modified at the head, tail and linker and tested their cytotoxicity against extracellular *L. donovani* promastigotes and intracellular *L. donovani* amastigotes using strains typed as antimony (Sb) resistant (Sb-R) or Sb-sensitive (Sb-S) individually or in combination. The most effective compound was then used in *in vivo* studies

to determine its ability to clear spleen, liver and bone marrow *L. donovani* parasites. Our results confirmed that the anti-leishmanial activity of APCs was dependant on their structure and that synergism was exhibited by some combinations.

Methods

Reagents

Giemsa stain was purchased from Sigma-Aldrich (Gillingham, UK). Penicillin/streptomycin, glutamine, medium 199, DMEM, RPMI-1640, PBS pH 7.4, penicillin/streptomycin, glycine and foetal calf serum were obtained from Invitrogen, Paisley, UK. All other reagents including the APCs were analytical grade and obtained from Anatrace, via their distributor Generon Ltd, Slough, UK, Sigma Aldrich, Poole, UK and gifted by Dr Mohamed Yaseen, UWS, UK.

Animals and parasites

Age-matched inbred BALB/c female mice (20 g-25 g) were used in studies at Strathclyde University. Animal studies were carried out with local ethical approval and had UK Home Office approval (project licence PPL60/4334). *L. donovani* cloned strains with different Sb susceptibility backgrounds were derived from isolates obtained from VL patients at the B.P. Koirala Institute of Health Sciences, Dharan, Nepal: MHOM/NP/02/BPK282/0cl4 (Sb sensitive, Sb-S). MHOM/NP/02/BPK087/0cl11 (Sb intermediate, Sb-I) and MHOM/NP/02/BPK275/0cl18 (Sb resistance, Sb-R [13]). In addition, the Sb sensitive strain (MHOM/ET/67:LV82) was also used in studies. Luciferase expressing promastigotes for the LV82 and Nepalese strains were prepared using previous published methods and the integrative construct (a gift from Dr D.F. Smith), designated pGL1313, contained pSSU-int fragments to facilitate integration into the ribosomal DNA locus of *Leishmania* (Alsaadi *et al.,* 2012[14]). *Leishmania donovani* LV82 (MHOM/ET/67:LV82), the Nepalese clinical *Leishmania donovani* isolates were cultured in complete RPMI 1640 medium (medium supplemented with 20% (v/v) heat-inactivated foetal calf serum, 1% (v/v) penicillin/streptomycin, 100 µg/ml and 1% (v/v) L-glutamine) at 25°C. Amastigotes were cultured within bone marrow derived macrophages from BALB/c in Dulbecco's modified eagle medium (DMEM supplemented with 20% (v/v) FCS, 1% (v/v) pen/strep (100 µg/ml), 30% (v/v) L-cell supernatant and 1% (v/v) L-glutamine) at 37°C, 5% CO_2.

In vitro cytotoxicity against *L. donovani*

The anti-leishmanial activity of APC against *L. donovani* luciferase-expressing promastigotes was determined by adding of the appropriate 100 µl parasite line (10^6 cells/ml) to the wells of a 96 well plate and adding 100 µl medium alone (controls) or 100 µl of APC compound (0.01 µg/ml - 6.25 µg/ml, n = 3/treatment). The plate was incubated for 72 h at 25 °C at 27°C. In combination assays, the additional compound/compounds, at a final concentration of 0.195 µg/ml or 0.39 µg/ml, was added to the initial APC being tested. In macrophage studies the method described used in previous studies was followed [14]. Briefly bone marrow derived BALB/c macrophages ($1\text{-}2\times10^5$) in 100 µl complete RPMI 1640 medium were added to the wells of a 96 well plate and left to adhere for 24h at 37°C, 5% CO_2 /95% air. The medium was removed and 100 µl of the appropriate *L. donovani* luciferase-expressing promastigotes line (1×10^6- 1×10^7) was added to each well. The plate was incubated for a further 24h, the medium removed, and 100 µl of the medium alone (control) or appropriate APC compound was then added (0.01 µg/ml - 0.197 µg/ml, n = 3/treatment). In combination assays, the additional compound/compounds, at a final concentration of 0.024 µg/ml or 0.048 µg/ml, was added to the initial APC being tested. All plates were incubated for 72h at 37°C, 5% CO_2 /95% air. Wells with macrophage and with no drug or promastigotes added were used in control experiments. Luciferin solution (1 µg/ml in 20 µl medium without FCS) was added to the appropriate wells of the 96 well plate at the end of the experiment and the amount of light emitted/well was measured using a luminometer (Biotek Synergy HT, relative light units) using a wavelength/bandwidth of 440/40 nm, or IVIS® imaging (Spectrum Living Image system®, total flux, photons/sec). The effect of drug treatment on parasite survival was determined by calculating the mean suppression in the light emitted from the drug treated experimental sample compared to the mean control value and used to calculate the IC_{50} using Prism GraphPad® software (version 5.0). The effect of drug alone on the viability of uninfected cells was determined using the same experimental protocol above but cell viability was determined using a colormetric alamar blue assay [15]. At the end of the incubation period cells 10 µl resazurin solution (0.02% w/v) was added to control and drug treated cells and the absorbance of samples was read at 575 nm and 595 nm. The effect of drug treatment on cell survival, which correlates with the magnitude of dye reduction, was expressed as percentage viability (or alamar blue reduction [16] and determined using the formula provided in the manufacturer's protocol:

$$\%\ alamar\ blue\ reduction = \frac{(\varepsilon_{ox}\lambda)(A\lambda_1) - (\varepsilon_{ox}\lambda_1)(A\lambda_2)}{(\varepsilon_{red}\lambda_1)(A'\lambda_2) - (\varepsilon_{red}\lambda_2)(A'\lambda_1)} x100$$

In the formula, $\varepsilon\lambda_1$ and $\varepsilon\lambda_2$ are constants representing the molar extinction coefficient of alamar blue at 575 nm and 595 nm, respectively, in the oxidized (ε_{ox}) and reduced (ε_{red}) forms. $A\lambda_1$, $A\lambda_2$ and $A'\lambda_1$, $A'\lambda_2$ represent absorbance of test and negative control wells at 575 and 595 nm, respectively. The values of % alamar blue reduction (or % viability) were corrected for background values of negative controls containing medium without cells. Percent viability values were used to calculate the IC_{50} using Prism GraphPad® software (version 5.0). The selectivity (SI_{50}) indices were determined as the quotient of the IC_{50} of uninfected macrophages compared to infected macrophages; and the resistant (RI_{50}) was determined as the quotient of the IC_{50}s of drug-resistant strain (Sb-I or Sb-R) to the drug-sensitive strain (Sb-S) respectively. Drug interaction profiles were determined using combenefit software® [17].

In vivo* cytotoxicity against *L. donovani

BALB/c mice were infected by intravenous injection (tail vein, no anaesthetic) with 1-2 × 10^7 *L. donovani* strain LV82 amastigote parasites, obtained from the spleen of an infected hamster. Mice (n = 4/treatment) were treated with PBS pH 7.4 (controls) or APC12 (40 or 80 mg/kg) by intravenous injection (tail vein, no anaesthetic, 0.2 ml) on days 7 and 8 post-infection or a single oral dose on day 7 post-infection. Milt was not given orally at a dose of 80 mg/kg because of its potential toxicity. On day 14 parasite burdens in the liver, spleen and bone marrow were determined [14]. Results are from duplicate experiments, however, if a treatment had no significant effect then it was tested in subsequent experiments.

Result

The effect of varied alkyl carbon chain lengths of APCs against *Leishmania donovani*.

Three APC series with physical modifications at the head, tail and linker (Fig. 1) were screened for their antiparasitic activity against *L. donovani* (MHOM/ET/67:LV82). The IC_{50} and IC_{90} values showed that promastigotes were more resistant to the Milt-analogue APC16 (mean IC_{50} ± SDS, promastigote stage, 0.70 ± 0.00 µg/ml, amastigote stage 0.10 ± 0.01 µg/ml; mean IC_{90}, promastigote stage, 6.3 ± 0.10 µg/ml, amastigote stage, 0.90 ± 0.016 µg/ml). The activity of APC16 against uninfected macrophages was much higher, and based on the IC_{50} values gave a selectivity index of 50 for the intracellular amastigote stage (Table 1 and Fig. S1). Decreasing the alkyl carbon chains of this molecule by two carbons produced APC14 and APC12, increased the biological activity of the resulting compound significantly along the series ($p < 0.01$) and peaked with APC12 (IC_{50} and IC_{90} of 0.163 ± 0.00µg/ml, 0.009 ± 0.00 µg/ml, and 1.47 ± 0.00 µg/ml, and 0.081 ± 0.01 µg/ml for promastigotes and intracellular amastigotes

respectively; Table 1). Molar concentrations of all compounds are detailed in Table 1. Decreased alkyl APC carbon chain length was synonymous with decreased hydrophobicity and increased readiness to form micellar structures that form pores on cell membranes to produce death by leakage [18]**.** Increasing hydrophobicity of APC12, by introducing a double bond between the first and second carbon atom on the APC12 tail (APC11UPC; Fig.1), allowed the formation of lamellar structures and not micelles [18] significantly reduced efficacy relative to APC12 (Table 1 and Fig. S1). Similarly, the addition of two alkyl carbon chains to the choline backbone of APC12, each with six alkyl carbons atoms, to reduce their ability to form micelles and increased hydrophobicity (APC11PC; Fig. 1 [18]) also significantly reduced efficacy relative to APC12 (Table 1 and Fig. S1). Intriguingly, death of *L. donovani* promastigotes and intracellular amastigotes judged by their IC_{50}s, occurred below, near and above the threshold concentrations micelles are formed also called the critical micellar concentration (CMC) for APC12, APC14 and APC16 respectively (Table 1 and Fig. S1). These results suggested that APCs with reduced hydrophobicity or increased ability to form micelles were effective anti-leishmanials.

Next, the antiparasitic activity of APC12, APC14 and APC16 against three Nepalese *L. donovani* clinical isolates with different inherent susceptibilities to antimony was tested. We found that the Sb-S, Sb-I and Sb-R [13] strains were also killed by the APC analogues and that efficacy was influenced by alkyl carbon chain length. APC16 and APC12 were the least and most toxic with death occurring below CMC for APC12- and APC14-treated Sb-S and Sb-I promastigotes, and above CMC for APC16-treated Sb-R promastigotes and amastigotes (Table 2 and Fig.S2). Resistance indexes (RI_{50}) for promastigotes and amastigotes ranged from 0.48-1.77 and 0.77-2.33 respectively, showed that the RI_{50} value reflected antimony resistance of the strain (Table 2; Fig. S2). Further, the selectively index (SI_{50}) showed that APC12 and APC14 were more effective than APC16 and were safe compounds for *in vivo* experiments in *Leishmania* mice models (Table 2). All of the compounds were more active against the LV82 *L. donovani* laboratory strain compared to the Nepalese clinical isolates (Compare Table 2 and Table 1), which may reflect the temporal difference in when these strains were isolated.

The effect of charge on tailed molecules against *Leishmania donovani*

Modifications to the APC12 head, by removing the P-atom, to produce a cationic amphiphile, where the N-atom attached to a one or two 12 alkyl carbons to produce DA and DAB respectively (Fig. 1), resulted in similar activity to APC12 based on IC_{50} values for DAB

against the amastigote and promastigote stages (Table 1). The anionic amphiphile, formed by the removal of the N-atom from APC12 to produce PO (Fig.1) was inactive against *L. donovani* compared to controls (Table 1).

The effect of charge separation on tailed molecules against *L. donovani*

Increasing the number of alkyl carbons in the linker between the N- and P-atoms in APC12 from two to six (APC12P6C; Fig.1) significantly decreased the compound's anti-leishmanial activity against both promastigotes and amastigotes relative to APC12 ($p < 0.01$; Table 1 and Fig. S1).

Studies to determine if APC can act synergistically against *L. donovani*

Combination of drugs with multiple targets can produce more effective antimicrobials and the discovery that APCs induced death below, near or above CMC against *L. donovani* allowed us to investigate their interaction as mixed APCs namely, APC12 with APC14 or APC16 by applying the Loewe additivity model to viability data using Combenefit [17]. APC12 mixed with APC14 interacted predominantly synergistic against promastigotes (Fig. 2A; Fig. S3) but was antagonistic against amastigotes intracellularly within macrophage (Fig. 2C, Fig. S4). In contrast, APC12 and APC16 had significant antagonistic interactions against both life cycle forms (Fig. 1B and 1D; Fig. S3 and S4) suggesting that identification of synergistic combination ratios was not a function of their physical properties, unpredictable and a systematic approach was required for identification.

In vivo* efficacy of APC12 against *L. donovani

In this study we decided to assess the *in vivo* anti-leishmanial activity of the most active analogue, APC12, in a murine model of VL. A dose at 40 mg/ml caused a significant reduction in parasite numbers in the spleen ($p < 0.05$) and liver ($p < 0.01$) but not in the bone marrow given by the intravenous route but had no significant activity when given by the oral route (Table 3). Doubling the drug dose administered by the oral route to 80 mg/kg still did not result in a significant reduction in parasite burdens in all three sites (Table 3). In contrast, oral treatment with Milt at a dose of 40 mg/kg resulted in a significant reduction in parasite numbers in both the spleen ($p < 0.05$) and liver ($p < 0.01$, Table 3). These results probably indicate that APC12 has poor bioavailability by the oral route.

Discussion

In this study we have shown that the activity of APCs against *L. donovani* was dependent on its alkyl carbon chain length, with a 12-alkyl carbon analogue that is zwitterionic or cationic (DA) charge on the head being most effective. The candidate APC12 was 10-fold and 4-fold more active than the milt-analogue, APC16 against promastigotes and intracellular amastigotes respectively. This phenomenon is pathogen specific, as a parallel study using the same compounds against the parasitic protist, *Acanthamoeba*, showed that APC16 was the most active [19]. Studies from elsewhere have also produced mixed results. For example, short and long chains APCs were most effective in reducing tumour development and against fungal infections respectively [20-23]. The reason for this is speculative and possibly depends on the pathogen's membrane infrastructure; where membranes with fatty acids with long chains are easily disrupted with complementary APCs. For example, the predominant fatty acids present in membrane phospholipids of *Acanthamoeba* trophozoites and *Leishmania* promastigotes are ~28-30 carbons and 30-40 carbon atoms respectively [24,25], and these are most sensitive to APC16 [19]. Nevertheless, the link between the number of the alkyl carbon chains in APCs and their CMCs and hydrophobicity is well established [26, 27]. Decreasing alkyl carbon chain length (APC16-APC12), results in alteration in the physical properties such as hydrophobicity and CMC [26]. Introducing unsaturation (APC11UPC) produced the expected results except for widening the charge distance from 33Å to 70Å to produce the flexible molecule, APC126PC. The reduced CMC for APC126PC was accompanied by a higher biological activity, possibly, because the flexibility produced at the head, affected its orientation and packing density on the biological membranes [28,29]. There are specific but limited studies on this theme to support this premise. For example, erucylphosphocholine [30], edelfosine [30], ilmofosine [31] and perifosine [32,33,34], with 21, 18, 16, 18 alkyl carbon atoms tails modified with a cis double bond, oxygen, sulphur or a piperidine ring at the head respectively, have produced more effective antileishmanial activity against *Leishmania spp* compared to Milt. These differences are perhaps dictated by their ability to form micelles at a lower critical micellar concentration (CMC; [28,29,36].

Nevertheless, the molecules used in this study produced death below (APC12), near (APC14) and above (APC16) the CMC. In bacteria, this produced three different death mechanisms [37], but evidence for this in *Leishmania* is limited. However, if this were the case for *Leishmania*, we postulate that mixtures of APCs with different and not similar CMC-APC

relationship should be synergistic. Combination treatment with APC12 and APC14 or APC16 was only synergistic for promastigotes and not amastigotes, which could reflect differences in drug uptake by macrophages or the ability to reach the intracellular parasites within the host cell. Generally, APCs mixtures can produce unique molecular species with physical characteristics such as surface tension, osmotic pressure, solubility and ease to form micelles significant different from their individual constituents [38-40]. It shows that studies should focus on the intracellular amastigote stage as this is what is clinically relevant and that we need to understand how drug combination function to produce better combination treatments. These type of regimens are very important as liposomal amphoptericin B and milt combination treatment acted synergistically giving a cure rates or >95% [41-44].

Our *in vitro* screening studies identified APC12 as the most potent compound but this high activity was not reflected in a murine VL model when administered orally. This suggests that the intestinal epithelium may be a significant barrier for oral absorption of APC12. Generally, the alkyl chain and zwitterionic head group in hydrophilic APCs, e.g. APC12 and APC16 modulates the activities of transporters e.g. P-glycoprotein, the human intestinal peptide transporter (PepT-1) and the monocarboxylic acid transporter (MCT-1) and the intercellular spaces between tight junctions (paracellular transport in membranes to navigate through the lipid bilayer of Caco-2 cell monolayer to increase bioavailability [45-49]. Based on this information, the reason for the intestine of mice to be a formidable barrier against the delivery of APC12 into systemic circulation is not readily apparent. Nevertheless, drug bioavailability of drugs are influenced by the characteristics of the site where the compound is absorbed and for the gut, factors such as (a) the absorbing surface area, bacterial flora, motility, pH, mucus thickness and food intake [50], (b) drug concentration [47-47] and (c) structure [51] are compounding factors. For example, Milt can be non saturable and saturable in a two-component population pharmacokinetic model below and above a threshold concentration of 50 μM (20.4 μg/ml) [45-49] while the ability of a series of 2-alkoxy-3-alkylamidopropylphosphocholine derivatives to alter cell membrane fluidity was a function of the lengths of their alkyl carbon chain lengths and not their ability to form micelles [51]. Nevertheless our study and previous studies, shows that oral treatment milt at 40 mg/kg (this study) or 25 mg/kg on days 7-11 [52] produced significant reduction in *L. donovani* parasite burdens for WT (this study; $p < 0.0010$) and a Sb sensitive and a Sb resistant strain, [52]. Further studies are required to investigate the non-translational nature of our *in vitro* cytotoxicity assays in our *in vivo* mice model.

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Figure legends

Fig. 1. Diagram to show the changes made to Milt to produce three APC series. The one tailed hexadecylphosphocholine, with sixteen alkyl carbon atoms on the tail (n-16, m-0, designated APC16) was progressively reduced by the removal of two alkyl carbons to give tetradecylphosphocholine, APC14, and dodecylphosphocholine, APC12. To APC12, a cis double bond was added between the first and second atoms, 10-Undecylenyl-1-phosphocholine, (APC11UPC) or its 12 alkyl carbon atoms reduced to two five alkyl carbon chains (n-5, m-5) attached to a carbon on the phosphoryl group to produce 2, 8-Dimethyl-5-Nonylphosphocholine (APC11PC). The positive and negative charge on the N- and P- atoms respectively were separated using four alkyl carbon atoms to give APC12P6C, or the N-atom (trimethyl amine moiety) or P-atom was removed to give dodecylamine, DA and PO respectively. Twelve alkyl carbons were added to produce didodecyldimethyl ammonium bromide (DAB). Significant structural changes are shown in red.

Fig. 2. Contour map of the interaction based on antileishmanial activity between APCs mapped-out with the Loewe model. The Combenefit software produced an interaction profile for APC12 (0.02-6.25 µg/ml for promastigotes (a,b) and 0.001-0.19 µg/ml for amastigotes (c,d) mixed with APC14 or APC16 at 0.195 µg/ml or 390 µg/ml for promastigotes and 0.02 µg/ml or 0.04 µg/ml for amastigotes. Synergistic (blue), additive (green to yellow) and antagonism (red) interaction were noted.

Author contribution statement

Authors contribution are as follows; Conceptualization, RAMW. and KCC; Methodology, RAMW, KCC; Formal Analysis, RAMW, KCC, HA; Writing, RAMW, KCC, HA; Supervision, RAMW, KCC; Project Administration, RAMW;

Conflict of Interest

No competing financial interests to declare

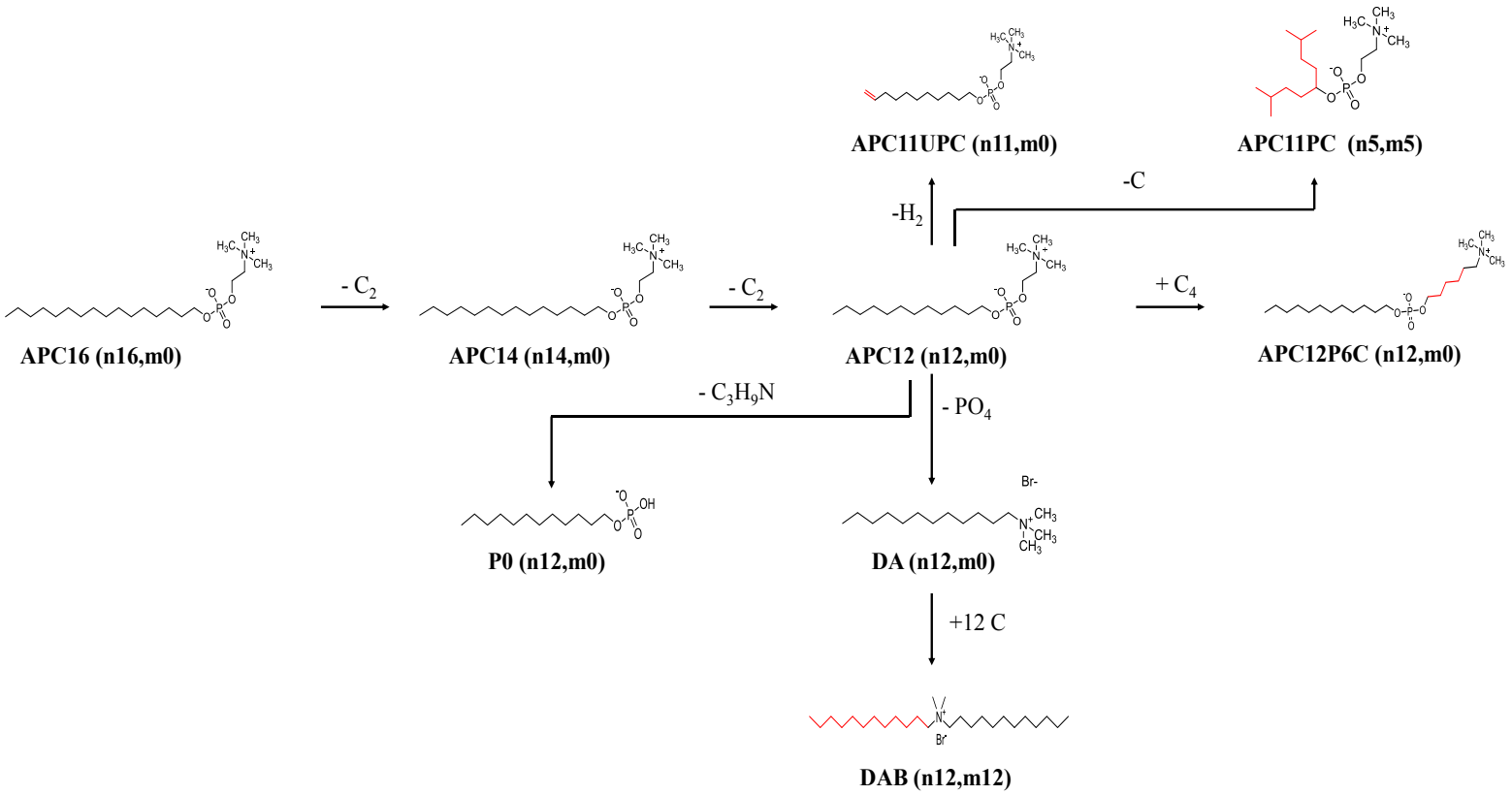
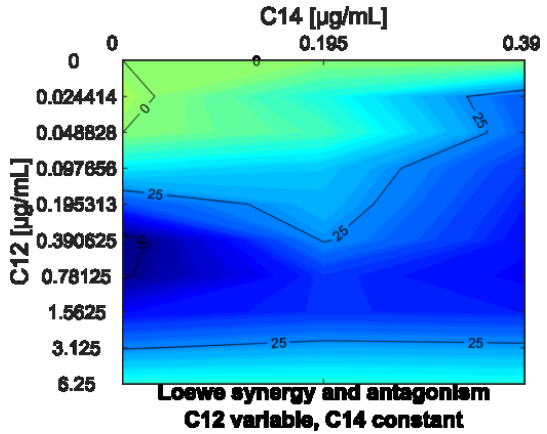
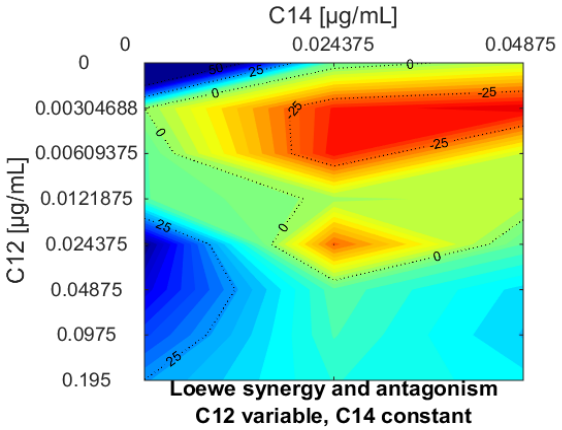

APC11UPC (n11,m0)
APC11PC (n5,m5)
-H2
-C
APC16 (n16,m0)
- C2
APC14 (n14,m0)
- C2
APC12 (n12,m0)
+ C4
APC12P6C (n12,m0)
- C3H9N
- PO4
P0 (n12,m0)
DA (n12,m0)
Br-
+12 C
DAB (n12,m12)

Fig. 1

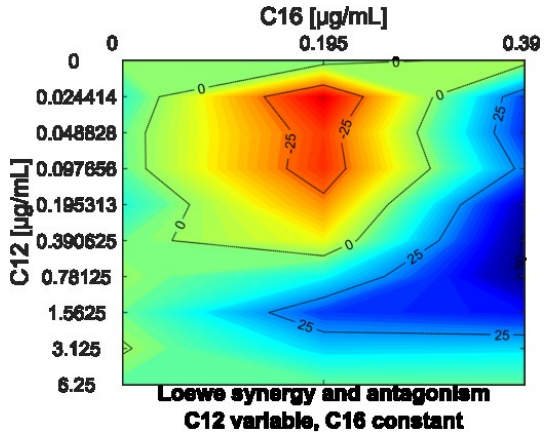
A


C14 [µg/mL]
0
0.195
0.39
C12 [µg/mL]
0
0.024414
0.048828
0.097656
0.195313
0.390625
0.78125
1.5625
3.125
6.25
Loewe synergy and antagonism
C12 variable, C14 constant

C


C14 [µg/mL]
0
0.024375
0.04875
C12 [µg/mL]
0
0.00304688
0.00609375
0.0121875
0.024375
0.04875
0.0975
0.195
Loewe synergy and antagonism
C12 variable, C14 constant

B


C16 [µg/mL]
0
0.195
0.39
C12 [µg/mL]
0
0.024414
0.048828
0.097656
0.195313
0.390625
0.78125
1.5625
3.125
6.25
Loewe synergy and antagonism
C12 variable, C16 constant

D

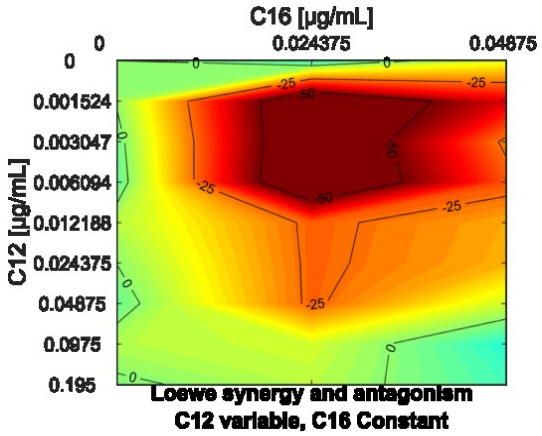

C16 [µg/mL]
0
0.024375
0.04875
C12 [µg/mL]
0
0.001524
0.003047
0.006094
0.012188
0.024375
0.04875
0.0975
0.195
Loewe synergy and antagonism
C12 variable, C16 Constant

Fig. 2

Table 1. IC_{50} and Selectivity index (SI_{50}) of APCs against *L. donovani* promastigotes, intracellular amastigotes and bone derived macrophages.

Compound	Chain length and number (n,m)	Charge	Promastigotes µg/ml (µM)	Amastigote µg/ml (µM)	Macrophage µg/ml (µM)	Selectivity index (SI_{50})	Promast:amast ratio	Molecular mass	CMC µg/ml (µM)
Modification of tail									
APC12	n-12; m-0	Zwitterionic	**0.16±0.00 (0.46±0.00)	**0.009±0.00 (0.026±0.00)	35.38±1.74 (100.64±4.95)	3535.35	16.46	351.5	351.50 ($^{+}$1.00)
APC14	n-14; m-0	Zwitterionic	**0.20±0.00 (0.53±0.00)	**0.03±0.02 (0.066±0.05)	40.89±4.42 (107.75±11.65)	1593.63	7.97	379.5	45.54 ($^{+}$1.2 x 10^{-5})
APC16	n-12; m-0	Zwitterionic	0.70±0.00 (1.72±0.00)	0.10±0.01 (0.25±0.02)	5.08±8.29 (12.47±20.34)	50.00	7.00	407.5	0.41 ($^{+}$1.3 x 10^{-5})
APC11PC	n-6; m-5	Zwitterionic	>6.25±0.06 (>18.63±0.18)	0.19±0.08 (0.55±0.24)	61.90±0.01 (184.56±0.03)	333.69	>33.60	335.4	N/A
APC11UPC	n-12; m-0	Zwitterionic	1.80±0.02 (5.37±0.06)	**0.01±0.06 (0.04±0.18)	54.00±0.13 (161.00±0.39)	4060.15	135.34	335.4	N/A
Modification of the linker									
APC12P6C	n-12; n-0	Zwitterionic	33.50±0.06 (82.21±0.15)	1.01±0.07 (2.47±0.17)	14.10±1.08 (34.60±2.65)	14.03	33.33	407.5	N/A
Modification of the head									
DA	n-12; m-0	Cationic	**0.09±0.00 (0.05±0.00)	0.47±0.00 (2.54±0.00)	37.60±0.01 (202.91±0.05)	79.83	0.18	185.3	N/A
DAB	n-12; m-12	Cationic	**0.04±0.05 (0.091±0.11)	**0.06±0.00 (0.119±0.00)	82.76±0.01 (178.91±0.02)	1514.65	0.79	462.6	N/A
PO	n-12; m-0	Anionic	>6.25±0.01 (>25.07±0.04)	>2.00±0.01 (>8.02±0.04)	100.00±0.01 (401.11±0.04)	>50.00	>3.12	249.31	N/A

*,**statistical decreased significance relative to APC16 at $p < 0.05$ and $p < 0.01$ respectively; $^{+}$

Table 2. IC_{50} and Selectivity index (SI_{50}) of APCs against *L. donovani* promastigotes and intracellular amastigotes with different inherent susceptibilities to antimony (Sb).

Strain	Antimony Resistance	Drug	Promastigotes µg/ml (µM)	Amastigotes µg/ml (µM)	Promast:amast	Resistant Index (RI_{50}) (Promast/amast)	Selectivity index (SI_{50})
282/4	**Sb sensitive**	APC12	**0.55±0.02 (1.56±0.06)	**0.26±0.01 (0.74±0.03)	**2.09**		136.08
		APC14	** 0.44±0.07 (1.16±0.18)	**0.12±0.02 (0.32±0.05)	**3.64**		340.75
		APC16	0.84±0.05 (2.06±0.12)	0.31±0.02 (0.76±0.05)	**2.68**		16.39
087/11	**Sb intermediate**	APC12	** 0.42±0.02 (1.19±0.06)	**0.20±0.07 (0.57±0.20)	**2.11**	0.76/0.77	176.90
		APC14	** 0.21±0.00 (0.55±0.00)	**0.19±0.01 (0.50±0.03)	**1.05**	0.48/1.58	215.21
		APC16	1.49±0.6 (3.66±1.47)	0.29±0.01 (0.71±0.02)	**5.15**	1.77/1.76	17.52
275/18	**Sb resistant**	APC12	** 0.49±0.03 (1.39±0.09)	**0.23±0.01 (0.65±0.03)	**2.10**	0.89/0.88	153.83
		APC14	**0.37±0.05 (0.97±0.13)	**0.28±0.00 (0.74±0.00)	**1.31**	0.84/2.33	146.04
		APC16	1.26±0.06 (3.09±0.15)	0.51±0.00 (1.25±0.00)	**2.49**	1.50/1.65	9. 96

*,**statistical decreased significance relative to APC16 at $p < 0.05$ and $p < 0.01$ respectively

Table 3 The *in vivo* activity of different formulations against *L. donovani* spleen, liver and bone marrow parasite burdens. *L. donovani* infected mice (n = 4 or 5) were treated with medium alone (intravenous route), miltefosine (Milt, oral), or APC12 (oral or intravenous route, IV) on day 7 post-infection and parasite burdens then assessed on day 14 post-infection. The mean percentage suppression ± SD in parasite burdens is shown in parentheses. *p < 0.05, **p < 0.01 vs control, [a]p < 0.05, [b]p < 0.01 MIL vs APC12, [c]p < 0.05 APC12 40 vs 80 mg/kg

Treatment	**Mean parasite burden ± SD**		
	Spleen	**Liver**	**Bone marrow**
Experiment 1: Oral administration			
Control	198 ± 62	1000 ± 286	225 ± 100
MIL 40 mg/kg oral	73 ± 68* (53 ± 31)	433 ± 46** (40 ± 25)	197 ± 157 (30 ± 35)
APC12 40 mg/kg oral	275 ± 96[b] (8 ± 18)	880 ± 276[a] (36 ± 34)	210 ± 90 (54 ± 34)
Experiment 2: Intravenous administration			
Control	305 ± 97	1299 ± 158	458 ± 129
APC12 40 mg/kg IV	112 ± 30* (60 ± 11)	434 ± 197** (60 ± 19)	555 ± 254 (8 ± 14)
APC12 80 mg/kg oral	257 ± 73[c] (13 ± 27)	939 ± 170[c] (27 ± 16)	607 ± 115 (2 ± 4)