Drug combinations are effective anti-leishmanials against drug resistant *Leishmania mexicana.*

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Abstract

Leishmania is a parasite that causes the disease leishmaniasis, and 700,000 to 1 million new cases occur each year. There are few drugs to treat the disease and drug resistance in the parasite is limiting the clinical utility of existing drugs. One way to combat drug resistance is to use combination rather than monotherapy. In this study we have compared the effect of single and combination treatment with four different compounds i.e. alkylphosphocholine analogues APC12 and APC14, miltefosine (MIL), ketoconazole (KTZ), and amphotericin B (AmpB), on the survival of *Leishmania mexicana* wild-type promastigotes and a cell line derived from the WT with induced resistance to APC12 (C12Rx). Combination treatment with APC14 and APC16 had a synergistic effect in killing WT while KTZ and APC12 or APC14 or APC12 and APC14 had synergistic effect against C12Rx. More than >90% killing occurred using APC12 alone at >1mg/ml against C12Rx strain, however combinations with APC14 produced similar killing using APC12 at 0.063mg/ml-0.25mg/ml and APC14 at 0.003mg/ml-0.5mg/ml. These results show that combination therapy can negate induced drug resistance in *L. mexicana* and that using this type of screening system could accelerate the development of drug combinations for clinical use.

Introduction

Leishmaniasis a disease caused by infection with the protozoan parasite *Leishmania* and 700,000 to 1 million new cases occur each year[1]. There are three clinical forms of leishmaniasis, cutaneous leishmaniasis, visceral leishmaniasis (VL) and mucocutaneous leishmaniasis. Development of preventive strategies such as vaccines and vector control are challenging and drug overuse and misuse has selected a plethora of resistant parasites in clinics, with most drugs having limited clinical efficacy in some cases[2]. Subversion of the therapeutic activity of anti-leishmanial by the parasite is due to the parasite's genomic and metabolic plasticity and gene switching, regulated by epigenetic and post-translational modifications[3-5]. The nature of drug resistance genes vary, and drug combinations administered simultaneously and sequentially have been shown to circumvent acquired resistance[6]. For this reason, drug combinations are often used for treatment for drug resistant conditions, for example cancer therapy[7-9]. However identification of what drugs or drug regimen should be used is often done on an *ad hoc* basis rather than having a cohesive strategy to identify optimal drug combinations and treatment regimens. Joint treatment with paromomycin (PMM) and miltefosine (MIL) can delay the selection of PMM resistance[11,11] in *L. donovani*, and joint treatment with pentavalent antimonial with PMM and liposomal AMB is recommended for VL in some countries[12]. For a widespread application of this approach, elaborate hypothesis-free, high-throughput screening assays of all possible physiological dose-ratio matrixes in pair-wise cytotoxicity assays with distinctive endpoints are required. This approach aids the mapping out of synergistic, additive, and antagonistic interaction profiles with mathematical models based upon the median-effect principal, combination index theorem or Loewe additivity and Bliss models, aided by online software such as Combenefit. The output from these models can inform the identification of molecules that target compensatory pathways used by drug resistant cells[7,13-15].

Many molecular targets have been described in *Leishmania*, and the biosynthetic pathway for its main membrane sterol ergosterol, synthesised from leucine[16,17] e.g. sterol 14α-demethylase (14α-DM; EC1.14.13.70) have been described as essential for parasite development[18-21]. Further, changes in the composition and proportion of sterols in most

drug-resistant parasites, even with molecular targets unrelated to sterol biosynthesis, point towards this pathway having an essential role in developing drug resistance[22-28] particularly in the biosynthesis of membrane phospholipids of *Leishmania* parasites[29-31]. Thus we hypothesise that the link between selection of resistant parasites and metabolic and genetic plasticity suggest that inhibitors that simultaneously target these biosynthetic pathways could be effective anti-leishmanials, particularly against drug resistant *Leishmania* parasites. To this end, we have developed a systematic proof-of-concept study to evaluate the cytotoxicity of two-drug combinations that targets sterols and phospholipids of *Leishmania mexicana* wild-type (WT) parasites and a cell line with laboratory induced resistance to the MIL analogue APC 12[32].

Results and Discussion

C12Rx promastigotes have altered sterol composition and are sensitive to sterol biosynthetic inhibitors.

It well known that drug-resistant *Leishmania*-parasites have altered levels of proteins involved in sterol biosynthesis of and intermediate and end products[22-26,28,30,33,34]. For example, sterol 14α-DM in *Leishmania amazonensi*s and sterol C-22 desaturase (EC 1.14.19.41) in *L. donovani* are upregulated in parasites resistant to ketoconazole (KTZ)[26] and amphotericin B (AmpB)[23], whilst stigmasterol is a potential biomarker for AmpB resistance in *L. donovani*[22]. Therefore the sterol profile of WT and a daughter strain **C12Rx**, which had laboratory induced resistance to the alkylphosphocholine analogue, **APC12**[32], was compared using logarithmic (log) and stationary growth phase promastigotes using gas chromatography-mass spectrometry (GC-MS). Five sterols, namely squalene, zymosterol, ergosterol, brassicasterol and cholesterol were identified in samples based on retention time, molecular weights, and electron impact mass spectra. The abundance of squalene and brassicasterol in WT were low at log and stationary phase respectively relative to cholesterol, zymosterol and ergosterol (Figure 1, dark gray bar). Squalene accounted for 0.01% of the total sterol in log stage promastigotes but increased to 0.19% in stationary phase promastigotes. Ergosterol and zymosterol were the major sterol in log stage parasites (ergosterol 62%, zymosterol 22%, Figure 1A, dark gray bar) and continued to be the major constituents in stationary phase promastigotes (ergosterol 68%, zymosterol 17%;

Figure 1B, dark bar and Table S1). Cholesterol which cannot be synthesised by *Leishmania* but is salvaged from their serum-supplemented (10% v/v) growth media, used for *in vitro* growth[35], increased in abundance (Figure 1, dark gray bar), between log and stationary growth phase *L. mexicana* WT, constituted 15% and 17% of the total sterol respectively. In APC12 resistant strain, C12Rx, ergosterol continued to be the major sterol present in log and stationary stage parasites, and was significantly reduced when compared to WT ($p < 0.01$).

The C12Rx strain took up significantly more cholesterol at stationary phase growth phase compared to the WT ($p < 0.01$), perhaps, to compensate for the loss of ergosterol, since cholesterol can only be derived from the culture medium[36,37]. Similar experiments in complete and spent medium from WT and C12Rx produced inconsistent results, probably reflecting the detection limit of the assay. Brassicassterol abundance was significantly different between log and stationary phase (Figure 1, light gray bar, $p < 0.01$), constituted 5% and 1% respectively. Interestingly, the brassicassterol to ergosterol conversion rate was significantly higher in the C12Rx strain compared to the WT for log phase promastigotes (WT, 3.11%; C12Rx, 6.92%) but equivalent for stationary phase parasites (WT, 2.71%; C12Rx, 2.48%), suggesting an altered sterol 17β-hydroxylsterol dehydrogenase (EC 1.1.1.51) activity[35]. The abundance of all 5 sterols were significantly higher in stationary than log phase cells for both strains ($p<0.01$) being 1.7-fold and 1.7-fold higher in WT (log, $2.12 \times 10^9 \pm 5.99 \times 10^7$; stationary, $6.46 \times 10^9 \pm 6.28 \times 10^7$) than C12Rx (log, $1.25 \times 10^9 \pm 4.78 \times 10^7$; stationary, $3.93 \times 10^9 \pm 8.10 \times 10^7$) strain at log and stationary growth phase respectively (Table S1). Overall, this data showed that APC12 resistance was associated with altered sterol profiles. This reduced sterol level in the C12Rx strain suggested that C12Rx was heavily reliant on its reduced intracellular sterols for survival, so we used sterol biosynthetic inhibitors (SBIs) to test the druggability of enzymes in this pathway.

Activity of Sterol Biosynthetic Inhibitors against *L. mexicana*

The importance of the enzyme sterol 14α-demethylase in parasite survival was determined by determining the sensitivity of the WT and C12Rx *L. mexicana* parasites to ketoconazole, KTZ, a specific inhibitor of this enzyme[38]. The APC12 strain was more sensitive to KTZ treatment as it had a significantly lower IC_{50} and IC_{90} ($p < 0.05$, mean $IC_{90} \pm SD$, WT, IC_{90} 60.36 ± 4.21μg/ml, C12Rx, 4.40 ± 0.48μg/ml; mean IC_{50} values shown in Table 1).

Amphotericin B, AmpB binds to and removes ergosterol from membranes and induces cell death in *Leishmania* parasites by altering the integrity of their cell membrane[39]. Treatment with AmpB was significantly more effective against the APC12 resistant strain compared to the WT ($p < 0.05$, mean IC_{90} ± SD, WT 9480 ± 474.28 µg/ml, C12Rx 5196 ± 24.23 µg/ml, mean IC_{50} values shown in Table 1). Although the C12Rx strain had a significantly higher endogenous cholesterol level at the stationary phase of parasite growth compared to the WT, this did have a protective role[36]. As cholesterol uptake has been deemed protective, our results for C12Rx, indicated that the higher drug-induced death than WT occurred whilst parasites were in the log phase of growth and cholesterol level reduced (compare Table 1 and Figure 1).

Table 1. Mean IC_{50} of SBIs against *L. mexicana* strains. WT or C12Rx promastigotes (5 x 10^6/ml, n=3 independent treatment in triplicate) at log phase were cultured in the presence of medium alone (controls) or different concentrations of ketoconazole (KTZ) or amphotericin B (AmpB) for 3 days at 25°C. The effect of treatment on parasite survival was determined by determining the mean suppression in parasite growth for each experimental value compared to the mean control value. This data was then used to determine the mean IC_{50} value using the Grafit software. The resistant index RI_{50} was the quotient of the mean IC_{50} values of two strains with C12Rx and WT being the numerator and denominator respectively. The p value compares IC_{50} data for WT vs. C12Rx strain.

SBIs	**IC_{50} µM (µg/ml)**		**Resistant Index (RI_{50})**	**p value.**
	WT	**C12Rx**		
KTZ	37.86 ± 0.62 (20.12 ± 6.65)	9.03 ± 0.50 (4.8 ± 1.97)	0.24	$p < 0.05$
AmpB	3419.62 ± 52.96 (3160 ± 232.12)	1899.19 ± 99.50 (1755 ± 13.28)	0.56	$p < 0.05$

Activity of alkylphophocholines against *L. mexicana*

Previous studies have shown that (a) phospholipids composition is different between miltefosine-, MIL-resistant and WT parasites for *L. donovani*[2]; and (b) the degree of cytotoxicity of APCs against *L. mexicana* was dependent on their alkyl chain length; with APC12 being more active than APC16 against naïve parasites, WT and C12Rx[32]. In this study we determined the activity of a 14 alkyl carbon chain, APC14, against both WT and C12Rx *L. mexicana* promastigotes to determine if cross-resistance occurred (Table 2). The dose response curves for the three APCs for WT and C12Rx are presented in Figure 2. The resistant indices obtained from the IC_{50}s of the APCs indicated that cross resistance did occur within the series but its effect was limited for APCs with longer alkyl carbon chains (Table 2). Death from APCs is caused by solubilisation of the parasite's membrane including *Leishmania,* leading to the formation of individual or aggregated molecules called micelles[30]; the latter are formed above a threshold concentration called the critical micellar concentration (CMCs). For example, MIL (APC16) at concentrations below, at or above the CMC has distinctive properties for example, promoting fluidisation of model membranes, loss of vesicle integrity and formation of mixed aggregates[30,37]. Extrapolation of this to the APCs used in this study, showed a structure-activity relationship, indicating that all the APCs tested were micellar-independent against the WT (CMC>IC_{50}; Table 2) but micellar formation was required for APC14 and APC16 against the C12Rx strain (IC_{50}>CMC; Table 2). These results suggest that membrane phospholipids metabolism or composition was altered in the C12Rx strain compared to the WT parent. Alteration of fatty acid and sterol metabolism has been described previously in miltefosine-resistant *Leishmania donovani* promastigotes[30].

Table 2. The activity of APCs against *L. mexicana.* WT or C12Rx promastigotes (5 x 10^6/ml, n=3 independent treatment in triplicate) were cultured in the presence of medium alone (controls) or different concentrations of the alkylphosphocholines, APC12, APC14 and APC16 for 3 days at 25°C. The effect of treatment on parasite survival was determined by determining the mean suppression in parasite growth for each experimental value compared to the mean control value. This data was then used to determine the mean IC_{50} value using the Grafit software. * micellar, **no micellar requirement; The resistant index RI_{50} was the quotient of the mean IC_{50} values of two strains with C12Rx and WT being the numerator and denominator respectively; CMC, critical micellar concentration.

Compound	IC_{50} μM (μg/ml)		p value	RI_{50}	CMC (μM)
	WT	C12Rx			
APC12	0.19 ± 001** (0.07 ± 0.03)	189.58 ± 10.14** (66.64 ± 12.26)	$p < 0.05$	952.00	1000*
APC14	0.72 ± 0.02 ** (0.27 ± 0.05)	130.96 ± 6.12* (49.70 ± 7.23)	$p < 0.05$	184.07	120*
APC16	4.32 ± 0.09x ** (1.76 ± 0.20)	70.92 ± 6.78* (28.90 ± 9.97)	$p < 0.05$	16.42	10*

***See Anatrace product description sheet for respective APCs**

Activity of mixed APCs against *L. mexicana*

Combination therapies are now a standard treatment for infectious diseases such as tuberculosis, HIV/AIDs and drug resistant parasites[40-43]. The success of this approach is due differences in the intracellular distribution drug profiles, their pharmacokinetic[44], pharmacodynamics[44] and the probability that the genes that confer parasite's resistant belong to the same molecular pathway or if from different pathways are not genetically linked[6]. We therefore assessed the ability of different APC combinations to inhibit the

survival of *L. mexicana.* APC12 mixed with APC14 (Figure 3A) or APC16 (Figure 3B), interacted predominantly antagonistically against WT parasites. In contrast, APC14 and APC16 had significant synergistic interactions (Figure 3C) suggesting that identification of synergistic combination ratios required a systematic approach and could not be predicted. Similar studies using C12Rx showed that APC14 and APC16, interacted antagonistically (Figure 3F) whereas, APC12 and APC16 and APC12 and APC14 acted antagonistically (Figure 3E) and synergistically (Figure 3D) respectively. These results confirmed the findings from WT, i.e. the physical properties of the individual APCs were not a key determinant to decipher their interaction profiles. Biophysical properties such as surface tension, osmotic pressure, solubility, packing on membranes and ease to form micelles of other tailed compounds will also have an impact. For example, cetyl trimethylammonium bromide (CTAB) and alkyltrimethylammonium bromide (CnTAB) mixture formed novel molecular species different from their individual counterpart[45-47]. Interestingly, treatment with APC12 and other APCs which interact synergistically negated the inherent resistance of the C12Rx strain (Figure 1D).

The effect of combination treatment with APCs and KTZ and AmpB was also investigated. KTZ mixed with APC12 or APC16 gave a synergistic effect (Figure 4A and 4C) but this did not occur for APC14 (Figure 4B). Joint treatment with individual APCs and AmpB had an antagonistic effect (Figures 4D-4F). Previous studies have shown that treatment with MIL (APC16) and AmpB produces pharmacokinetic antagonism as AmpB dissociates into monomers and formed mixed aggregates which cannot penetrate through membranes[46]. Although our studies indicate that APC-AmpB mixture in our assays contained molecular species which were impermeable to the membrane of *Leishmania* promastigotes, liposomal AmpB and miltefosine have been successfully used as an effective treatment for VL[48-50]. However, in this case entrapment of AmpB into vesicles meant that, the vesicle protected the drug from the external milieu and targeted the drug for uptake by infected macrophages[51,52].

Conclusion

Treatment with drug combinations that have a synergistic effect can increase the efficacy of a drug compared to monotherapy and be an approach that can be used to extend the clinical life of an existing drug[53]. Our phenotypic repurposing screen has allowed the identification of new synergistic APCs and SBIs drug combinations for the treatment of *L. mexicana*, and given a greater understanding of metabolic changes associated with APC resistance in the C12Rx strain. This approach can be used with other *Leishmania* species and extended to include the intracellular amastigote stage, which is the stage treated in the clinic. Use of suitably labelled parasites e.g. luciferase-expressing, would allow higher through-put screening[54] and help in drug repositioning studies using parasites with different inherent drugs sensitivities, so that any identified regimens are active against drug resistant parasites.

Experimental Section

Strain and Cultures: Transgenic *Leishmania mexicana* promastigotes (5×10^6 cells/ml) of strain MYNC/BZ/62/M379 expressing the firefly luciferase gene and sensitive to the MIL APC12 with 12 alkyl carbon chain called APC12 were designated WT; a related strain, C12Rx, resistant to 80µg/ml APC12, was selected under controlled conditions by a stepwise progressive increase of APC12 as described in Alotaibi, et al., 2019[32]. Both were cultured in complete Modified Eagle's Medium (M199 supplemented with 10% (v/v) heat inactivated foetal calf serum) at 25 °C. The transgenic line cultures were further supplemented with Hygromycin B in order to retain the extrachromosomal luciferase gene.

Gas chromatography mass spectroscopy (GC/MS) of *Leishmania* spp sterols

Samples used for this extraction were 10^9 cells/ml log phase (day 3) and stationary phase (day 6) cells pooled from several *in vitro* cultures to achieve the desired cell density with sterol abundance above the baseline of the GC-MS. Each pooled sample from an experiment was split into three replicates containing 10^9 cells and the sterols present extracted. Sterols were extracted using the sterol extraction kit done as detailed in the manufacturer's instruction (Sigma Chemical Co Ltd, Poole, UK). The extracted sterols were resolved and the analysed by GC/MS on the Thermo Scientific TRS-MS (5% (v/v) polar)

controlled by the Thermos Xcalibur software using the method described by Xu et al.[36]. Electron ionization mass spectra of major *Leishmania* sterols were performed at 70 eV. The retention time and total ion mass spectral compared with known standards in the NIST mass spectral library was used for sterol identification. Data are presented as mean ± SD for three separate experiments. The conversion of ergosterol to brassicasterol was calculated as the percentage of brassicasterol formed compared to the mass of brassicasterol + ergosterol extracted from the cells. Fold changes in total sterols were the quotient of the total sterol abundance in WT divided by that in C12Rx.

Compound efficacy studies: The anti-leishmanial activity of APCs against *L. mexicana* luciferase-expressing promastigotes cultured in complete M199 was determined by adding 100 µl of the appropriate parasite line (5 x 10^5 cells/ml) to the wells of a 96 well plate and adding 100 µl complete M199 alone (controls) or 100 µl medium containing the relevant compounds (APC, 0.01 µg/ml-6.25 µg/ml for WT and 0.01 mg/ml-4mg/ml for C12Rx, n=3/treatment), KTZ (6.25 µg/ml-0.06 µg/ml for WT and C12Rx) or AmpB (3.125 mg/ml-0.0003 mg/ml for WT and C12Rx). The samples were incubated for 72h at 25°C, long enough for nutrients in medium to be non-limiting in the no drug controls. After, 20 µl luciferin solution was added to the appropriate wells of the 96 well plate (1 µg/ml in medium without FCS), and the amount of light emitted/well was measured using a luminometer (Biotek Synergy HT, relative light units) at a wavelength and bandwidth of 440/40 nm. The effect of drug treatment on parasite survival was determined by calculating the mean suppression in the light emitted from the drug treated sample compared to the mean control value. The suppression data was then used to calculate the IC_{50} or IC_{90} value using Grafit software (version 5.0). The resistant index (RI_{50}) was the quotient of the mean IC50 values of two strains with C12Rx and WT being the numerator and denominator respectively. Drug interaction profiles were determined using published methods using Combenefit®[55]. Data are mean ± SD for three independent experiments carried out in triplicates.

Conflicts of interest

There are no conflicts to declare.

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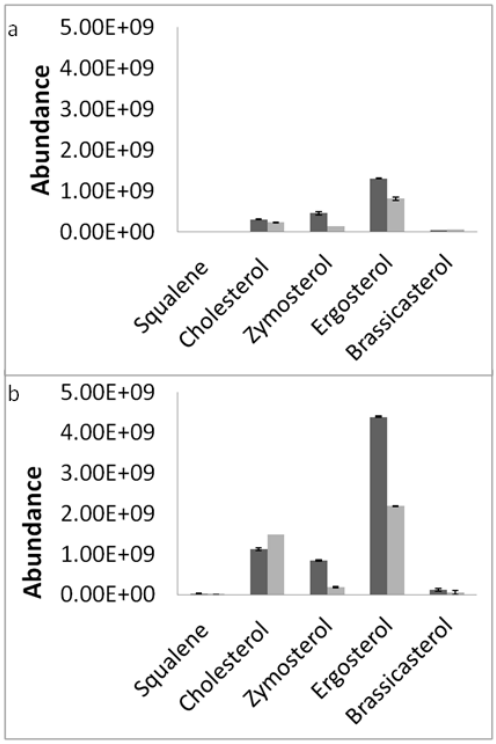

Figure 1. Sterol abundance in *L. mexicana* promastigotes. Sterols extracted from 10^9 WT (dark gray bar) and C12Rx (light gray bar) promastigotes at log phase (3 days in *in vitro* cultures from a starting cell density of $5x10^5$ cells; top panel) and stationary phase promastigotes (6, days in *in vitro* culture, bottom panel) estimated from the area under of the curve in chromatograms of the 5 sterols identified using authentic sterol after GC-MS analysis. Data are mean ± SD of sterols from three replicated from pooled cultures.

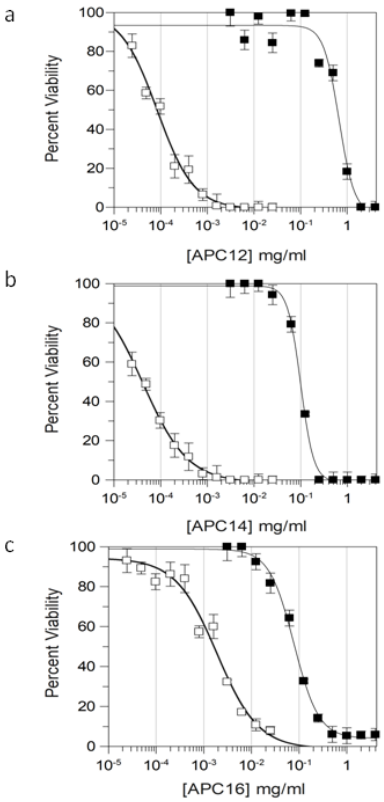

Figure 2. Dose response curves of alkylphoshocholine analogues against promastigotes of *Leishmania mexicana*. Promastigotes ($5x10^6$) of WT (open squares) and C12Rx (closed squares) incubated with the APC analogues named APC12 (top panel), APC14 (middle panel) and APC16 (bottom panel) at concentrations range: WT, $2.44x10^{-5}$ -0.025 µg/ml; C12Rx, 0.003mg/ml to 4mg/ml, for 72h at 26°C and cell viability assessed using the luciferase assay at wavelength/ bandwidth, 545/40 nm. Mean and standard deviation from three independent cytotoxicity assay each with three replicates were used to produce the dose response curve with the software 'Grafit' and IC_{50}s estimated.

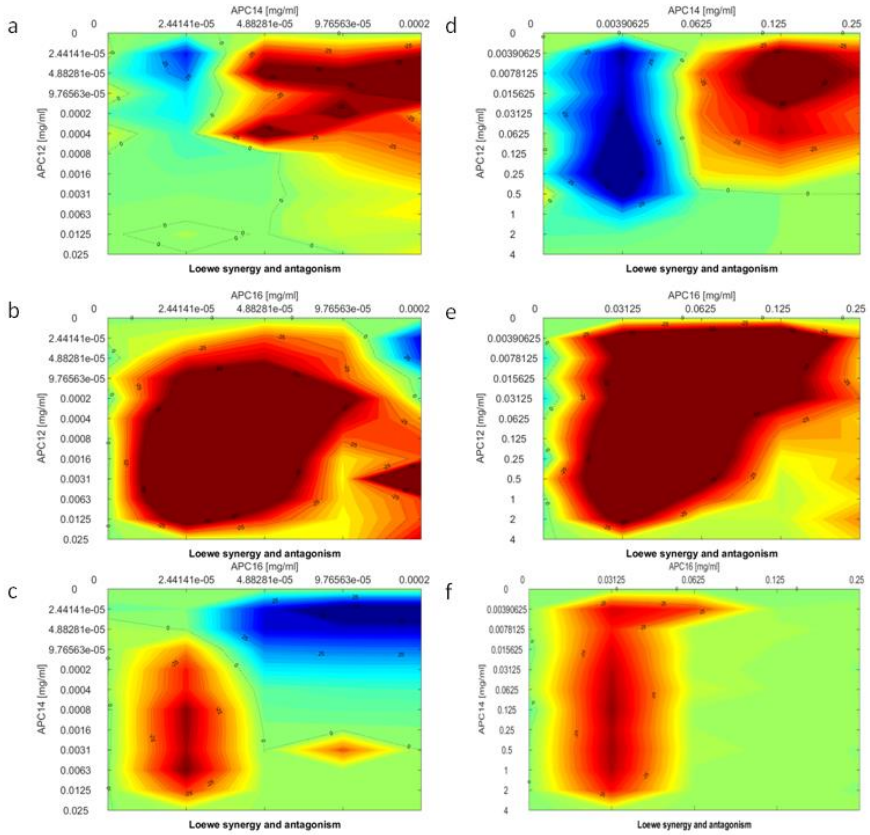

Figure 3. Contour map of the interaction based on anti-leishmanial activities between APCs mapped-out with the Loewe model. The Combenefit software produced an interaction profile for WT (left) and C12Rx (right) treated with APC12 and APC14 (A); APC12 and APC16 (B) and APC14 and APC16 (C). Drug concentrations for WT ranged from 2.44x10-5 - 0.025µg/ml while for C12Rx, it was 0.003mg/ml to 4mg/ml. Synergistic (blue), additive (green to yellow) and antagonism (red) interaction were noted.

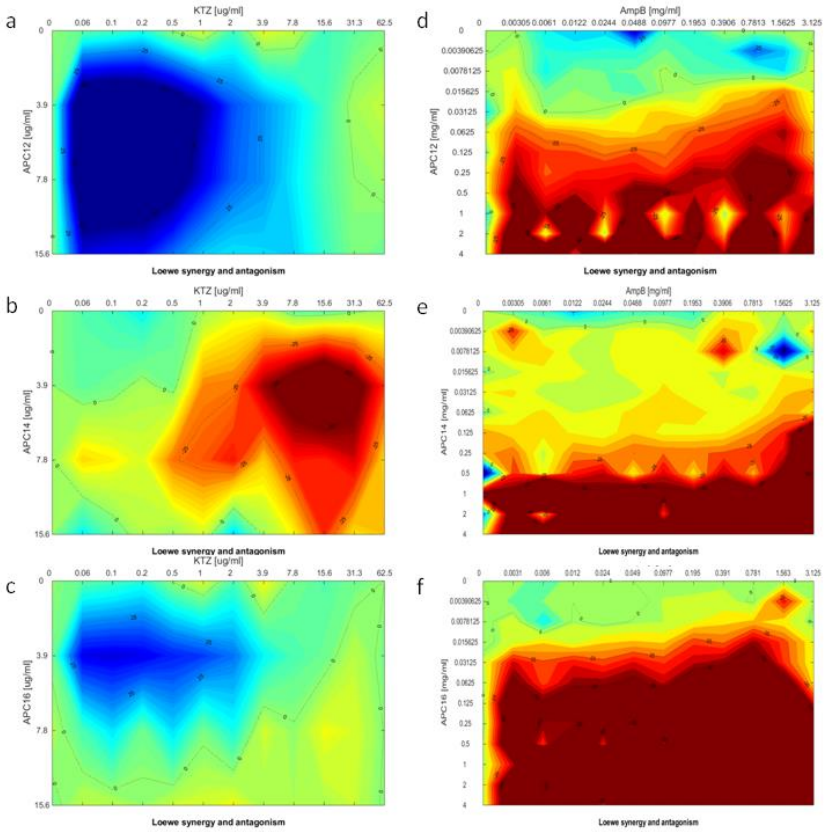

Figure 4. Contour map of the interaction based on anti-leishmanial activities of APCs and sterol biosynthetic inhibitors, KTX (left) and Amp B (right) mapped-out with the Loewe model. The Combenefit software produced an interaction profile for C12Rx treated with APC12 (A, D) APC14 (B, E) or APC16 (C, F) and KTZ (A, B, C) or Amp B (D, E, F). Drug concentrations for APCs ranged from 0.003mg/ml to 4mg/ml; KTZ from 6.10x10-5mg/ml - 0.0625 mg/ml) and Amp B from 0.003mg/ml – 3.125mg/ml. Synergistic (blue), additive (green to yellow) and antagonism (red) interaction were noted.