



## *In vitro* activity and *in vivo* efficacy of a combination therapy of diminazene and chloroquine against murine visceral leishmaniasis

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### Abstract

The present study evaluated the *in vitro* activity and *in vivo* efficacy of diminazene combined with chloroquine as a potential drug against *Leishmania donovani*. Amphotericin B was used as a positive control drug. *In vitro* activity involved incubation of various drug concentrations with promastigotes or vero cells in culture before determination of parasite growth inhibition or cell death while *in vivo* evaluations involved infection of various mice groups with virulent *L. donovani* parasites and treatment with test drug compounds following disease establishment. Weight changes in experimental mice were also evaluated before infection and throughout the experiment. The results indicated that the diminazene-chloroquine combination was at least nine times more efficacious than individual drugs in killing promastigotes in culture. The diminazene-chloroquine combination was safer ( $Ld_{50}=0.03 \pm 0.04$ ) than Amphotericin B ( $Ld_{50}=0.02 \pm 0.01$ ). Body weight in infected mice increased significantly ( $P=0.0007$ ) from day 7 to day 37 following infection ( $P=0.026$ ). However, body weight remained comparable in all mice groups during treatment ( $P=0.16$ ). The diminazene-chloroquine combination significantly reduced splenic parasite numbers as compared to individual drug therapies ( $P=0.0001$ ) although Amphotericin B was still more efficacious than any other treatment ( $P=0.0001$ ). Amongst the test compounds, the diminazene-chloroquine combination showed the lowest level of IgG antibody responses with results indicating significant negative correlation between antileishmanial antibody responses and protection against disease. These findings demonstrate the positive advantage and the potential use of a combined therapy of diminazene-chloroquine over the constituent drugs. Further evaluation is recommended to determine the most efficacious combination ratio of the two compounds.

**Keywords:** efficacy, diminazene-chloroquine, combination therapy, *Leishmania donovani*, visceral leishmaniasis, BALB/c mice

### Introduction

Visceral leishmaniasis (VL), also known as Kala-azar, is a protozoan systemic infection, which is always

fatal if left untreated<sup>[1]</sup>. The parasite is transmitted to humans through the bite of female sandflies that have previously fed on an infected reservoir. The disease is associated with anaemia, fever, weight loss, bone

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marrow destruction and hepatosplenomegaly<sup>[2]</sup>. It may cause epidemic outbreaks with high mortality. There is currently no vaccine against any form of leishmaniasis in routine use anywhere in the world<sup>[3]</sup>. Vector control measures, such as residual insecticide spraying and the use of insecticide-treated bed nets or curtains may offer effective protection<sup>[4]</sup>. However, treated bed-net programs are poorly implemented in many endemic countries and are beyond the means of many families in endemic villages. Current control is based on chemotherapeutic treatments which are expensive, toxic and associated with high relapse and resistance rates<sup>[1,5]</sup>. In the development of new drugs, combination therapy of antileishmanial drugs is currently considered as one of the most rational approaches to lower treatment failure rate and limit the spread of drug resistance<sup>[6]</sup>. Diminazene containing compounds have been tested and found to have trypanocidal and leishmanicidal activities on various strains of trypanosomes (*Trypanosoma vivax*, *T. evansi* and *T. congolense*) and on *L. donovani* in hamsters<sup>[7]</sup>. Studies conducted in the 1970s suggested the potential of berenil (diminazene diacetate) as a leishmanicidal agent<sup>[8]</sup>. When tested *in vitro* against both *Leishmania major* and *L. donovani*, Trypan<sup>®</sup>, a diminazene drug was found to be efficacious in controlling promastigote growth<sup>[9]</sup>. The same experiment also carried out *in vivo* studies with Trypan<sup>®</sup> against murine cutaneous leishmaniasis caused by *L. major* and the results showed delayed lesion development. A recent study has tested the synergistic effects of diminazene and artesunate (an anti-malarial drug) against murine visceral leishmaniasis and the results pointed to a potential application of this drug combination against leishmaniasis<sup>[5]</sup>. The results indicated that, diminazene contributed more to the drug combination potency than the effect of the artesunate and hence the need to explore diminazene further. Diminazene granules represent a new formulation, containing, in part, diminazene diacetate in addition to other new ingredients. The drug is formulated for both oral and parenteral administration.

Chloroquine is a cheap, relatively well tolerated drug initially developed for the treatment of malaria in the 1930s. Chloroquine has, however, since accrued a plethora of uses in the treatment and amelioration of several other diseases and conditions because of its lysosomotropic properties<sup>[10]</sup>. It also has characteristic physiological and systemic effects. *Leishmania*, being a protozoan parasite just like *Plasmodium*, there is likelihood that chloroquine can also be used as an antileishmanicidal drug agent, but this had not been tested scientifically. The synergistic effects of

diminazene and chloroquine had not been tested for its potential application against leishmaniasis. The present study investigated the protective potential of diminazene (Dim) combined with chloroquine (Chq) compounds in BALB/c mice experimentally infected with *L. donovani*. The *in vitro* safety and efficacy of the drug combination were also evaluated.

## Materials and methods

### Parasites for infection

*Leishmania donovani* strain NLB-065 originated from the spleen of an infected patient and was maintained by intracardiac hamster-to-hamster passage at the Institute of Primate Research. A hamster splenic aspirate was cultured in Schneider's *Drosophila* insect medium supplemented with 20% fetal bovine serum and 100 µg/mL of gentamicin at 25 °C till the stationary phase. Parasites harvested at the stationary phase of culture were centrifuged at 2500 rpm for 15 minutes at 4 °C and washed three times in sterile phosphate-buffered saline before being counted and used for the study.

### Chemicals

Diminazene diacetate and chloroquine granules were provided by Dr Alain Bourdichon (TropMed, Germany). The compounds were weighed separately, mixed in a 1:1 ratio by weight and used in dosages of 12.5 mg/kg of body weight. Chloroquine and diminazene were also individually used at dosages of 12.5 mg/kg. Amphotericin B at doses of 1.0 mg/kg was used as a positive control drug.

### Toxicological assay

Vero cells were cultured and maintained in minimum essential medium (MEM), supplemented with 10% fetal bovine serum. The cells were cultured at 37 °C in 5% CO<sub>2</sub>, harvested by trypsinization, pooled in a 50 mL vial and 100 µL cell suspension ( $1 \times 10^5$  cell/mL) put in to duplicate wells of rows A-H in a 96-well microtitre plate for one sample. The cells were incubated at 37 °C in 5% CO<sub>2</sub> for 24 hours to attach, the medium aspirated off and 50 µL of the highest concentration of each of the test samples serially diluted and added to appropriate test wells. The cultures were incubated further at 37°C for 72 hours. Surviving number of cells was used to calculate cell death per each well and results presented as percentage cell death as well as mean dose of drug compound or lethal dose (Ld<sub>50</sub>) that killed 50% of the original vero cells per test drug concentration.

### ***In vitro* evaluation against *Leishmania donovani* promastigotes**

Stationary-phase promastigotes harvested as described above were counted and suspended in a concentration of  $2.0 \times 10^6$  parasites/mL in culture medium. The tests were performed as previously described<sup>[11]</sup>. The tests were performed in 96-well microtitre plates maintained at 26 °C under 5% CO<sub>2</sub> atmosphere. Two hundred microliters of complete Schneider's Drosophila medium were placed in the wells containing the maximum concentrations of the compounds and 100 µL in the next wells (2 to 12) and controls; 2 µL of compound solutions of 20 mg/mL in distilled water were added to wells number 1 and serial dilutions (ranging from 100 µg/mL to 0.049 µg/mL) performed in the wells. Hundred microliters of culture medium containing  $2.0 \times 10^6$  stationary-phase *Leishmania donovani* parasites were added to each test well. Tests were performed two times each with three replications for each test compound concentration. Parasite observations and counting was done using a microscope. The results were expressed as the mean drug concentration inhibiting parasite growth by 50% (IC<sub>50</sub> ± SD) after 72 hours incubation period. The initial concentrations for testing were 100 µg/mL.

### ***In vivo* evaluation against *Leishmania donovani***

Six to eight week old BALB/c mice of both sexes were infected intraperitoneally with  $1 \times 10^6$  virulent *Leishmania donovani* parasites harvested at the stationary phase. Infected mice were kept for five weeks for symptomatic establishment of visceral leishmaniasis. Infection was assessed in three mice by splenic aspirate culture and impression smears. The animals were then divided into five groups of eight mice each and treated with Dim, Chq, Dim-Chq or Amphot B. One group was not treated and served as a control. A naïve (uninfected) group of mice was also included in the study. The test drugs were given at dosages of 12.5 mg/kg of body weight while the reference drug, amphotericin B was given at a concentration of 1 mg/kg of body weight. All doses were intraperitoneally administered consecutively for 21 days from week seven post infection.

### **Determination of parasite burden in *L. donovani* infected mice**

Two weeks following the last day of treatment, all infected mice were sacrificed and parasite numbers were determined microscopically by counting the

number of amastigotes in Giemsa stained splenic impression smears. Amastigote burdens were compared for both experimental and control groups and results expressed as the number of amastigotes per 500 splenic cell nuclei as described before<sup>[12]</sup>.

### **Monitoring of body weights**

Individual animal weights were taken before infections were done. In addition, the weights of both treated and non-treated mice groups were measured before, midway and at termination. Mice from each study group were weighed by placing individual mice in a beaker whose weight had been predetermined and then placing the setup on an electronic weighing balance. Reductions or increases in body weights were calculated from the differences of individual body weights taken before infections and weights measured during the course of treatments. Weights from the treated groups were compared with those from the control group to make evaluations on the effectiveness of the drug.

### **Enzyme-linked immunosorbent assay (ELISA)**

The assay was performed as described<sup>[13]</sup>. Briefly, polystyrene Micro-ELISA plates (Nunc, Copenhagen, Denmark) were coated overnight with 100 µL of *Leishmania donovani* soluble antigen at a concentration of 10 µg/mL, diluted in bicarbonate buffer (pH 9.6). Nonspecific binding sites were blocked with 3% bovine serum albumin (BSA) in PBS/0.05% Tween 20 buffer (washing buffer) for 1 hour at 37 °C. The plate was washed 6 times with washing buffer before the addition of 100 µL of the serum samples and incubation for 2 hours at 37 °C. The plate was washed 6 times as above and 100 µL of 1:4000 horse radish peroxidase-conjugated sheep anti-mouse IgG (Amersham) was used as detecting antibody. Tetramethlebenzidine (TMB) microwell peroxidase substrate was added to the wells and the plate was incubated in the dark for 20 minutes before the optical densities were read at 630 nm in a micro-plate reader (Dynatech Laboratories). All sera were tested at a dilution of 1:10, which had been previously determined as the optical dilution for antibody detection by titration.

### **Statistical analysis**

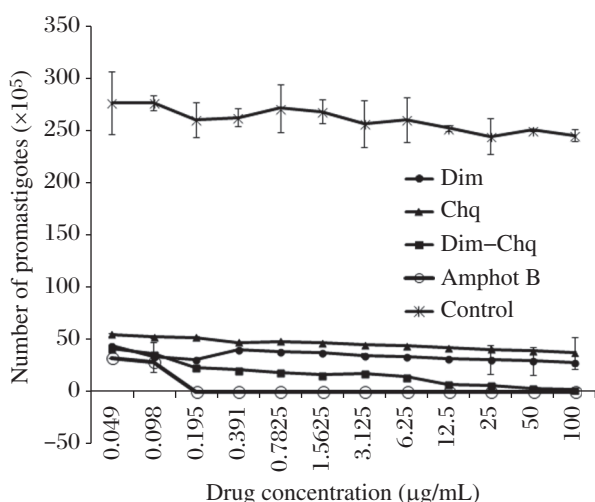
All parasite burden data were expressed as the mean ± SD per 500 cell nuclei of spleen cells. Differences among groups were analyzed by one-way analysis of variance (ANOVA), and the post hoc Tukey-Kramer test method was used for multiple comparisons. A *P*-value < 0.05 was considered statistically

significant. All analyses were performed using the Graph Pad InStat software.

## Results

### *In vitro* evaluation against *Leishmania donovani* promastigotes

Promastigotes incubated with various drug concentrations showed an upward increase in numbers with decrease in drug concentration. Amongst the test drugs, the combined drug, Dim-Chq, was more efficacious as compared to individual drugs. Incubation of promastigotes with Dim-Chq (100 µg/mL) reduced their numbers from  $2 \times 10^6$  to  $1.8 \times 10^5$  and the parasite numbers increased gradually to  $4.1 \times 10^6$  promastigotes when incubated with 0.049 µg/mL of Dim-Chq. The single drugs were less important in the control of promastigotes growth with Dim showing more inhibitory strength than Chq. However, none of the single drugs reduced the number of promastigotes below the initial starting numbers in any of the drug concentrations ranging between 100 µg/ml and 0.049 µg/mL. The reference drug, Amphot B, remained the most efficacious, killing all the promastigotes at all concentrations from 100 µg/mL to 0.195 µg/mL. The last two concentrations of Amphot B failed to kill promastigotes as there were  $2.8 \times 10^6$  and  $3.22 \times 10^6$  parasites in wells incubated with drug concentrations of 0.098 µg/mL and 0.049 µg/mL,



**Fig. 1** *In vitro* activity of drugs against *L. donovani* promastigotes. Promastigotes were incubated with various drug concentrations and parasite numbers counted 72 hours later to establish growth inhibition activities by the drug compounds. Data represents the mean numbers of viable parasites in wells at 72 hours of incubation. Dim: Diminazene, Chq: Chloroquine, Dim-Chq: Diminazene+Chloroquine, Amphot B: Amphotericin B.

**Table 1** Inhibition concentration 50 (IC<sub>50</sub>) values of various drug compounds against *L. donovani* promastigotes (mean ± SD)

Compound*	IC <sub>50</sub> (µg/mL)
Diminazene	4.254 ± 0.4
Chloroquine	5.85 ± 0.45
Diminazene+Chloroquine	0.49 ± 0.24
Amphotericin B	0.08 ± 0.01

respectively. Promastigotes in the control wells ranged from  $2.44 \times 10^7$  to  $2.76 \times 10^7$  indicating at least a 12 fold increase in numbers (**Fig. 1**).

A computation of drug concentration inhibiting parasite growth by 50% (IC<sub>50</sub>) indicated that Dim-Chq (IC<sub>50</sub>) was 9 times more effective than Dim (IC<sub>50</sub>) alone and 12 times more efficacious than Chq (IC<sub>50</sub>) alone in the control of promastigotes growth *in vitro* (**Table 1**). However, the positive control drug, Amphot B, remained more effective (IC<sub>50</sub>=0.08 ± 0.01) than Dim-Chq in inhibiting parasite growth as it showed 6 fold strength than the combination drug.

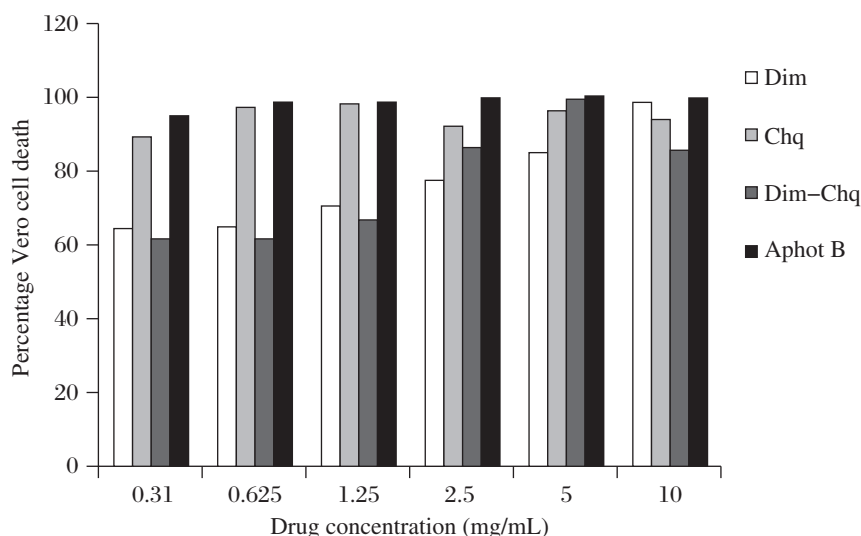
### Drug toxicity levels on Vero cells

Drug toxicity, scored as death of Vero cells following 72 hour incubation with the various drugs with concentrations ranging from 10 mg/mL to 0.31 mg/mL indicated that, the combination test compound, Dim-Chq or Dim alone killed fewer Vero cells as compared to Chq alone. Amongst the test drug compounds, Chq alone appeared the most toxic drug with a killing rate of at least 90% at each concentration tested. However, it was notable that Amphot B was the most toxic compound, killing over 95% of Vero cells at any given concentration (**Fig. 2**).

The minimum concentration or lethal dose 50 (Ld<sub>50</sub>) killing half the number of Vero cells indicated that both Dim (Ld<sub>50</sub>=0.03 ± 0.01 mg/ml) and Dim-Chq (Ld<sub>50</sub>=0.03 ± 0.24 mg/ml) were less toxic requiring to be in a concentration of 0.03 mg/mL to kill 50% of Vero cells in a given *in vitro* test system. Chloroquine alone and Amphot B showed the same

**Table 2** Ld<sub>50</sub> values of various test compounds and amphotericin B on Vero cells (mean ± SD)

Compound*	Ld <sub>50</sub> (mg/mL)
Diminazene	0.03 ± 0.01
Chloroquine	0.02 ± 0.05
Diminazene+Chloroquine	0.03 ± 0.04
Amphotericin B	0.02 ± 0.01



**Fig. 2 Percentage death of Vero cells following incubation with various concentrations of test drugs.** Vero cell lines were cultured with various drug concentrations for evaluation of drug toxicity. Data represents mean percentage death of Vero cells calculated at the end of incubation time. Dim-Chq drug combination was less toxic to the parasite based on the low dosage required to kill the Vero cells in comparison to the high dosage required to kill Vero cells. Dim: Diminazene, Chq: Chloroquine, Dim-Chq: Diminazene+Chloroquine, Amphot B: Amphotericin B.

toxicity strength at  $Ld_{50}=0.02$  mg/mL in killing 50% of Vero cells (**Table 2**).

### Weight changes in infected mice

All experimental mice groups showed increase in weights 37 days following infection with virulent *Leishmania donovani* parasites. Weight increase as a result of growth was calculated to be 0.77 in the naïve group of mice on day 37 following initial weight measurements. Paired t-test analysis indicated significantly higher weights seven days post infection than the baseline body weights ( $t=10.446$ ;  $df=5$ ;  $P=0.0007$ ). Following subtraction of 0.77 g from the mean weight taken from each group on day 37 after infection, and pairing of this new weight with the baseline weights measured before infection, it was interesting to note that, the paired t-test analysis indicated significantly higher ( $t=3.123$ ;  $df=5$ ;  $P=0.0262$ ) weight in each

treatment mouse group after infection with virulent *L. donovani* parasites (**Table 3**).

### Weight changes in mice following treatment

The mean weights of mouse groups at baseline and one week post treatment ranged from  $26.30 \pm 0.45$  g being the lowest taken from the Dim-Chq group to  $28.18 \pm 0.94$  g, being the highest taken from the Dim treated group. Weights from other treatment groups were intermediate (**Table 4**). A two tailed t-test for paired case analysis indicated that the pre- and post-treatment weights were comparable ( $t=1.715$ ;  $df=4$ ;  $P=0.1615$ ).

### Parasitic burden in splenic tissue

Two weeks following 21 day daily drug delivery into infected mice, amastigotes in splenic tissues from treated mice showed parasitic burden ranging from 1

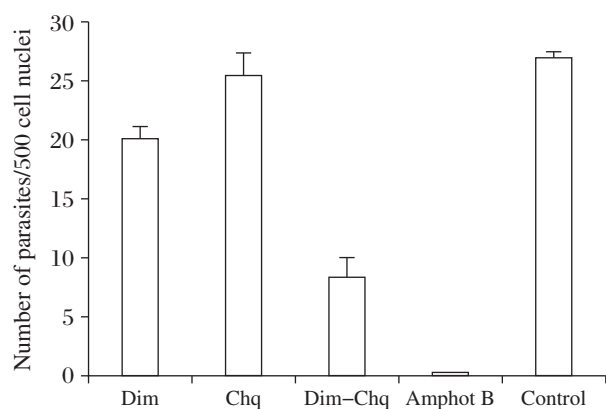
**Table 3 Weights of mice before and after infection**

Mice groups	Mean weight (g)		
	Days before and after infection		
	0	37	Day 37 -0.77g
Diminazene (Dim)	27.82	28.84	28.07
Chloroquine (Chq)	26.59	27.98	27.21
Diminazene-Chloroquine(Dim-Chq)	25.52	26.61	25.84
Amphot B	25.86	27.27	26.5
Control	26.11	27.02	26.25
Naïve (Not infected)	27.74	28.51	27.74

**Table 4** The weights of various treatment groups

Mice groups	Mean weight (g)		
	Days before and after treatment		
	0	7	Mean $\pm$ SD
Diminazene (Dim)	28.84	27.51	28.18 $\pm$ 0.94
Chloroquine (Chq)	27.98	26.91	27.45 $\pm$ 0.76
Diminazene-Chloroquine (Dim-Chq)	26.61	25.98	26.30 $\pm$ 0.45
Amphotericin B (Amphot B)	27.27	27.99	27.51 $\pm$ 0.51
Control	28.51	27.78	28.15 $\pm$ 0.52

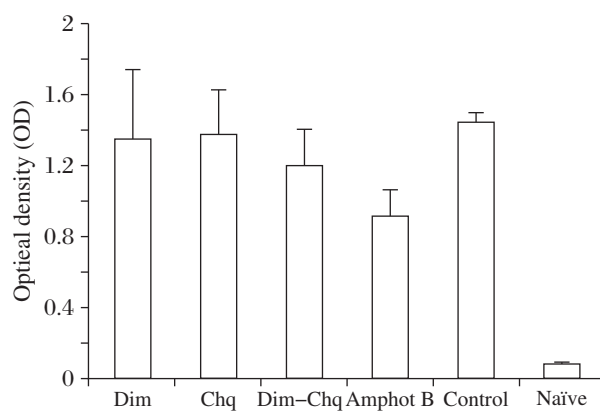
amastigote per 500 splenic macrophages in the Amphot B treated group to 28 amastigotes/500 cell nuclei in the control mice group (**Fig. 3**). Parasite numbers amongst the test drug treated mice were 9, 20 and 26 amastigotes/500 splenic cell nuclei in the groups treated with Dim-Chq, Dim and Chq, respectively. Analysis with ANOVA indicated a highly significant difference in parasitic numbers between any of the treated groups and the control group ( $F=158.81$ ;  $P=0.0001$ ). Significantly lower parasite numbers were associated with treatment with Dim-Chq than with either Dim or Chq alone ( $P=0.0001$ ). Between the single drug treatments, Dim treated mice showed significantly lower parasite numbers than Chq treated mice ( $P=0.001$ ). However, treatment with Amphot B was more protective than treatment with any other compound ( $P=0.0001$ ).



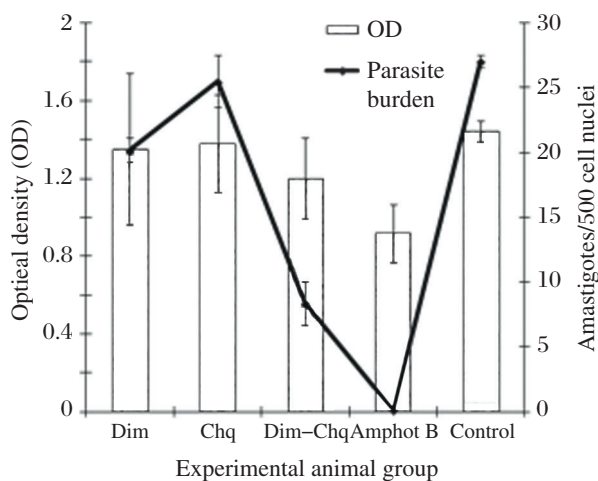
**Fig. 3** Number of amastigotes in infected macrophages in splenic impression smears. Groups of mice were infected with *Leishmania donovani* parasites and kept for five weeks for establishment of disease. From week seven post infection, infected mice were treated with various drug compounds for 21 consecutive days and splenic parasite numbers were determined two weeks following last treatment day. Dim-Chq combination drug therapy significantly reduced the parasite burden in mice compared to Dim or Chq single therapy. Data represents mean parasite numbers per 500 splenic cell nuclei. Dim: Diminazene, Chq: Chloroquine, Dim-Chq: Diminazene+Chloroquine, Amphot B: Amphotericin B.

### Antileishmanial antibody responses

All infected mice produced significantly higher antileishmanial IgG antibodies as compared to the naïve (uninfected) mice ( $F=2.848$ ;  $P=0.0361$ ). Amongst groups treated with the test drugs, Dim-Chq induced the least IgG antibodies followed by mice treated with Dim while treatment with Chq alone induced the highest antileishmanial antibodies. It was interesting to note that treatment with Amphot B was associated with lower IgG antibodies as compared to other drug treated groups as well as the infected untreated (control) mice. The control mice induced higher IgG antibodies than any other infected mice (**Fig. 4**). However, one-way analysis of variance (ANOVA) did not reveal any significant difference in IgG OD values for all experimental groups ( $F=1.450$ ;  $P=0.2528$ ).



**Fig. 4** Antileishmanial antibody responses in groups of mice following infection and treatment with various drug compounds. Groups of mice were infected with *Leishmania donovani* parasites and kept for five weeks for establishment of disease. From week seven post infection, infected mice were treated with various drug compounds for 21 consecutive days before sacrifice and determination of antibody responses two weeks later. Data represents mean optical density values  $\pm$  SD. Dim: Diminazene, Chq: Chloroquine, Dim-Chq: Diminazene+Chloroquine, Amphot B: Amphotericin B (n=8 mice per group).



**Fig. 5 Relationship between antileishmanial responses and parasite burden in mice following infection and treatments.**

Groups of mice were infected with *Leishmania donovani* parasites and kept for five weeks for establishment of disease. From week seven post infection, infected mice were treated with various drug compounds for 21 consecutive days before sacrifice and determination of antibody responses and splenic parasites two weeks later. Data represents relationship between mean parasite numbers and antibody responses  $\pm$  SD. Dim: Diminazene, Chq: Chloroquine, Dim-Chq: Diminazene+Chloroquine, Amphot B: Amphotericin B (n=8 mice per group).

### Relationship between antibody responses and parasite burden.

Spearman rank correlation analysis showed a positive significant correlation between antibody responses and amastigote numbers in treated and control mice ( $r=1.000$ ;  $P=0.0167$ ; **Fig. 5**). This relationship indicated that the group with strongest IgG antibody responses was associated with the highest number of amastigotes while the group with the lowest IgG responses was more protected against visceral leishmaniasis.

### Discussion

Combination therapy of antileishmanial drugs is currently considered as one of the most rational approaches to lower treatment failure rate and limit drug resistance spreading<sup>[6,14]</sup>. Furthermore, combination therapy between commercially available drugs that aims to reduce cost, toxicity and duration of treatments, represents a promising rational alternative<sup>[6]</sup>. As part of the efforts to develop safe and effective drug against leishmaniasis, the present study evaluated the *in vitro* safety and efficacy of this drug against *Leishmania donovani* promastigotes as well as the effects of the drug combination of visceral leishmaniasis in the murine system. Results on clinical disease outcome,

toxicity, antileishmania antibody (IgG) responses and efficacy are discussed.

The positive synergistic effects of Dim-Chq combination over the use of its constituent drug compounds in controlling the multiplication of promastigotes was demonstrated in culture. The more than 9 and 12 fold ability, of Dim-Chq over Dim and Chq alone, respectively to inhibit promastigotes growth by half the original numbers was expected because it is generally accepted that combination therapy is more effective than single drug therapies. In a recent *in vitro* study, diminazene combined with artesunate was shown to be at least two times more effective as compared to the single drug use in inhibiting *Leishmania donovani* promastigotes multiplication<sup>[5]</sup>. Other studies have reported the advantage of application of combined drug compounds over the use of the single drug chemicals in the development of drugs against leishmaniasis<sup>[6,14]</sup>. In the present study, the lower value of IC<sub>50</sub> for Dim than Chq indicates that Dim and not Chq must have contributed more to the potency of the combination drug compound. However, with the Amphot reference drug indicating a six fold strength in killing promastigotes by half the original number as compared to the combination drug, the *in vitro* parasite killing strength of the Dim-Chq is still low.

The extremely high toxicity levels associated with the Amphot B reference drug confirms the fears and limitations of use of current chemotherapy against leishmaniasis and the need for search of safer drugs that can invalidate the use of current toxic compounds. Indeed, current drugs for leishmaniasis including amphotericin B are highly toxic<sup>[5-6,15-16]</sup>. It was important to develop a drug that is completely safe, or at least, a drug that is safer than the Amphot B used as reference in this study. Associating the Dim-Chq compound with less toxicity levels was therefore a desired value, but it may be of concern that the Chq fraction in this combination therapy was as toxic as Amphot B. This Chq toxicity was unexpected based on recent reports that, in the treatment against human cutaneous leishmaniasis, intralesional application of Chq was found to be safe with no association with adverse events<sup>[17]</sup>. The higher toxicity levels associated with Chq than with Dim in the present report must have contributed to the lower LD<sub>50</sub> value for the combination therapy since Dim was safer when used alone. The safety of diminazene has also been reported in previous murine<sup>[9]</sup> and canine<sup>[18]</sup> *in vivo* studies. However, despite Dim and the combination therapy having the same toxicity levels, the combination of Dim and Chq would still be considered superior over Dim (or Chq) alone based on the extremely low dosage

required of Dim-Chq to kill half the number of disease causing pathogens as compared to the extremely high dosage required to kill half of a given number of cells. As a dosage ratio of 1:61 of the drug combination would be required to kill half the number of promastigotes and half the number of healthy cells, respectively, this combination therapy is still considered safe and can be developed further for control of leishmaniasis.

Infections with visceral leishmaniasis caused by *L. donovani* are associated with weight loss as one of the major symptoms of the disease<sup>[19]</sup>. In the present study, the significant increase in weight in all infected mice 37 days after *L. donovani* infection may be attributable to weight increases in the livers and spleens as a result of parasite multiplication. This may be the case, given that this weight increase was still significant in experimental mice in relation to naïve mice even after subtracting weight changes caused by normal body mass increase. This finding is supported by a recent study which recorded 14% and 32% weight increase in *L. chagazi* infected B2R7 C57BL/6 knock-out (KOB2) mouse livers and spleens, respectively<sup>[20]</sup>. The weight increase in mice used in the present study may be an early event in visceral leishmaniasis infected subjects before substantial damage to the viscera which subsequently results in weight loss.

The lack of a significant difference in the weights of various experimental and control groups following treatments may suggest the difficulty in evaluating drug efficacy based on body weight as a clinical parameter in this study. Weight loss is a symptom of progressive visceral leishmaniasis<sup>[21]</sup>. However, in the present report, the slight weight increase in mice treated with Amphot B and the slight reduction in weights in all other groups do not seem to give a clear indication of reliability on weight as a parameter to classify disease severity. Furthermore, the duration of this study from infection through treatment to termination of the experiment may not have been long enough for mice to develop severe disease where body weight would be significantly affected by disease severity.

The significant protection against visceral leishmaniasis in mice treated with Dim-Chq as opposed to Dim or Chq single drug therapy was an indication of the advantages of drug combination in the development of drugs against diseases. This observation may be partly due to different modes of action of combined drugs, which may effectively reduce parasite resistance. Dim appeared to have contributed more than Chq to the effectiveness of Dim-Chq in significantly reducing parasite numbers in mice treated with the combination therapy. This may be the case, given the many experiments that

have reported association of diminazene with high efficacy in the treatment of cutaneous leishmaniasis in murine and human subjects<sup>[5,9,22]</sup>. However, recently, in the treatment of human cutaneous leishmaniasis, intraleisional use of chloroquine appeared to be highly effective when compared to meglumine antimoniate<sup>[17]</sup>. It therefore follows that a combination of diminazene and chloroquine may be very effective for treatment of visceral leishmaniasis depending on the formulation ratio used. Indeed, the more effective compound can be used at a higher concentration than the less effective drug to formulate a combination with a desired efficacy. This is because based on the present findings, if parasitic burden alone was to be used to assess the importance of the combination therapy, the report would invalidate the use of this combination therapy against visceral leishmaniasis and recommend the use of Amphot B due to its significant ability to reduce parasite burden in infected mice.

The significant antileishmanial antibody responses observed in all *L. donovani* infected mice may have been an indication of active visceral leishmaniasis. This confirms earlier findings that presence of antileishmanial antibodies could be predictive of disease<sup>[23]</sup>. Furthermore, it has been reported that patients with active visceral leishmaniasis have high level of antileishmanial antibodies<sup>[24-27]</sup>. With this understanding, and considering the findings that in the present report, mice treated with different drugs induced different levels of IgG antibodies, it would be appropriate to suggest that antibody responses may be used for diagnosis and prognosis of visceral leishmaniasis. This is further supported by the high antibody responses associated with the non treated control mice infected with *L. donovani* parasites. Previous related studies have shown that, in mice, there is accumulating evidence that B cells and antibodies contribute to the visceral leishmaniasis pathology<sup>[28-29]</sup> and that mice lacking B cells were found to be less susceptible to *L. donovani* infection<sup>[30]</sup>. The significant correlation between antibody responses and parasite loads in study subjects was a confirmation that higher antibody responses are associated with severe disease. Previous studies have indicated that there is a correlation of high antibody titres during active disease and a fall in antibody levels following successful cure<sup>[31]</sup>. The present findings may be extended to mean that antibody responses may be reliably used as a diagnostic and/or prognostic parameter in visceral leishmaniasis. The lack of positive association between antibody levels and protection from disease confirms earlier findings that, antileishmanial antibodies, which are produced at low levels in cutaneous leishmaniasis and at very high levels in visceral leishmaniasis, play no role in protection and



that a high antibody level is a marker of progressive disease in visceral leishmaniasis<sup>[32-33]</sup>.

Based on the above results, it is clear that combination therapy is more potent than single drug treatments. The results have demonstrated the positive advantage and possible application of Dim-Chq drug combination in the safe and effective treatment of visceral leishmaniasis. It is possible that much of the efficacy of this drug combination is attributable to the presence of Dim and hence the need for further experimental design to establish the curative combination ratio and the *in vivo* toxicity parameters of these compounds.

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