Leishmania donovani Growth Inhibitors from Pathogen Box Compounds of Medicine for Malaria Venture

Introduction: Leishmaniasis is a collective term used to describe various pathological conditions caused by an obligate intracellular protozoan of the genus Leishmania. It is one of the neglected diseases and has been given minimal attention by drug discovery and development stakeholders to narrow the safety and efficacy gaps of the drugs currently used to treat leishmaniasis. The challenge is further exacerbated by the emergence of drug resistance by the parasites.

Methods: Aiming to look for potential anti-leishmanial hits and leads, we screened Medicines for Malaria Venture (MMV) Pathogen Box compounds against clinically isolated Leishmania donovani strain. In this medium-throughput primary screening assay, the compounds were screened against promastigotes, and then against amastigote stages.

Results: From the total 400 compounds screened, 35 compounds showed >50% inhibitory activity on promastigotes in the initial screen (1 μM). Out of these compounds, nine showed >70% inhibition, with median inhibitory concentration (IC_{50}) ranging from 12 to 491 nM using the anti-promastigote assay, and from 53 to 704 nM using the intracellular amastigote assay. Identified compounds demonstrated acceptable safety profiles on THP-1 cell lines and sheep red blood cells, and had appropriate physicochemical properties suitable for further drug development. Two compounds (MMV690102 and MMV688262) were identified as leads. The anti-tubercular agent MMV688262 (delamanid) showed a synergistic effect with amphotericin B, indicating the prospect of using this compound for combination therapy.

Conclusion: The current study indicates the presence of additional hits which may hold promise as starting points for anti-leishmanial drug discovery and in-depth structure–activity relationship studies.

Keywords: Leishmania donovani, Pathogen Box compounds, Medicines for Malaria Venture, drug discovery
generally been thought that *L. donovani* remains in Africa and India, whereas *L. infantum* was transported to South America and was named *L. chagasi*.

*Leishmania* parasites are digenetic organisms with a flagellated promastigote stage found in the insect vector and a non-flagellated amastigote stage found in the mononuclear phagocytic system of vertebrate hosts. The promastigote forms are found in the gut of the sand fly, then later migrate to the anterior portion and are inoculated into the dermis. Following infection, promastigotes invade macrophages and transform into amastigotes, where they undergo multiple asexual divisions until a host cell is packed with amastigotes and ruptures. Liberated amastigotes affect the reticuloendothelial system and associated organs that harbor macrophages.

From the estimated annual incidence of 2 million new cases of leishmaniasis, 25% are VL cases. More than 97 countries and territories are endemic for leishmaniasis, among which 65 countries are endemic for both visceral and cutaneous leishmaniasis. The disease is widely distributed around the world, ranging from the intertropical zones of America and Africa, and extending to tropical and subtropical areas of the Americas, southern Europe and Asia.

Chemotherapy is the only treatment option for VL. The choice of treatment for VL is often governed by regional practice in relation to what is currently most effective and available. The range of drugs available for the treatment of VL is limited. These include pentavalent antimonials (Sbv), amphotericin B deoxycholate, lipid formulations of amphotericin B (L-AB), miltefosine (MF) and paromomycin (PM), all of which have limitations in terms of safety, variable efficacy, inconvenient routes of administration and inconvenient treatment schedules, drug resistance and cost. Therefore, there is a high demand for new anti-leishmanial drugs which are safe, convenient for administration and affordable.

Developing new compounds, from initial target identification to final validation, takes more than 12 years and costs hundreds of millions of dollars. Therefore, short-term strategies such as searching for novel anti-leishmanial leads by phenotypic screening is an important approach to discover and develop drugs against leishmaniasis. Drug repurposing, compared to development of new drugs, is time efficient and cost effective. The art of drug repurposing usually starts with developing a method for screening and proceeds to hit identification, lead optimization and clinical studies. The present study exploits the phenotypic screening technique to identify anti-leishmanial “hits” from the Pathogen Box (PB) compounds.

The PB contains 400 pure compounds active against various neglected tropical diseases such as malaria, kinetoplastids, schistosomiasis, hookworm disease, toxoplasmosis and cryptosporidiosis. Screening these compounds for leishmaniasis is vital to maximize and exploit the richness of the PB to maintain momentum towards the discovery of new drugs. The Medicines for Malaria Venture (MMV) Pathogen Box has been screened for different protozoan parasites. Screening of PB compounds on *Trypanosoma brucei brucei* uncovered new starting points for anti-trypanosomal drug discovery. A study on *Neospora caninum* by Müller et al identified new compounds with profound activities. A screening by Hennessey et al came across three new inhibitors with dual efficacy against *Giardia lamblia* and *Cryptosporidium parvum*. PB screening on *Plasmodium falciparum* revealed one digestive vacuole-disrupting molecule. PB was also screened for *Toxoplasma gondii*, resulting in several novel compounds as a starting point for new drug discovery.

Here, we report findings from in vitro screening of MMV PB compounds against clinically isolated *L. donovani* promastigotes and amastigotes. The report is an independent analysis of the PB. We have also identified new compounds that could potentially serve as a starting point for new drug discovery in leishmaniasis.

**Methods**

**Test Strains, Cell Line, Laboratory Animals and Test Substances**

Clinical isolates of *L. donovani* strain were obtained from the Leishmaniasis Research and Diagnostic Laboratory (LRDL), Addis Ababa University. Strain AM1295 was isolated from a 33-year-old female patient residing in Liben district, Oromia region, Ethiopia, in 2017. Human monocytic leukemia (THP-1) cell line was kindly provided by Dr. Adane Mihret, Armauer Hansen Research Institute (AHRI). Peritoneal macrophages were harvested from Swiss albino mice obtained from Addis Ababa University animal house.

The PB was supplied in sealed plates containing frozen 10 mM dimethyl sulfoxide (DMSO) solutions in 96-well plates. Supplied compounds were further dissolved in DMSO and 10 copies were stored at −20°C as a stock. During the assay, the stock compounds were diluted in media.
**Leishmania Parasite Isolation and Culture**

*Leishmania donovani* isolates were cultured in NNN media and transferred to tissue culture flasks containing Medium 199 (M199) supplemented with 15% heat-inactivated newborn calf serum (HINBCS), 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2 mM L-glutamine, 100 IU/mL penicillin and 100 µg/mL streptomycin solution (all from Sigma-Aldrich, USA), and incubated at 26 °C.

**THP-1 Cell Line Cultures**

THP-1 cells were cultured in RPMI 1640 Medium (Sigma Aldrich, USA) supplemented with 10% HINBCS, 100 IU/mL penicillin and 100 µg/mL streptomycin at 37°C in a 5% CO₂ humidified incubator.²²

**Intraperitoneal Macrophage Collection and Culture**

Macrophages were collected from Swiss albino mice according to a method described by Zhang et al²³ with minor modification. Mice were injected with 2% freshly prepared starch into the peritoneal cavity. The inflammatory response was allowed to progress for 2 days, then the mice were killed. The skin underlying the peritoneal cavity was removed and the peritoneal cavity was washed with 10 mL of sterile ice-cold phosphate-buffered saline (PBS) with 3% HINBCS was injected into the peritoneal cavity. The peritoneal wall was massaged carefully, and macrophages were harvested by drawing 6–8 mL exudates of the PBS. The contents were transferred into sterile 15 mL test-tubes, and centrifuged at 450 g for 10 min. The resulting pellet was resuspended in minimum essential medium (MEM) containing 10% HINBCS, 25 mM HEPES, 2 mM L-glutamine, 100 IU/mL penicillin and 100 µg/mL streptomycin. After adjusting cell density to 1×10⁶/mL, 3×10⁵ macrophages were transferred to 24-well microculture plates.

**Anti-promastigote Assay**

Primary screening of the library consisting of 400 compounds against *L. donovani* was conducted according to methods described elsewhere²⁴,²⁵ with minor modifications. In brief, test compounds were diluted in 96-well microculture plates containing complete 100 µL M199 medium. Then, a 100 µL of suspension of the parasites (1×10⁶ promastigotes/mL) was added to each well to achieve the final concentration of 1 µM test compounds. Plates were incubated at 26°C for 72 h. Inhibition of parasite growth was determined using a resazurin-based fluorescence assay. The fluorescence intensity after a total incubation time of 72 h was estimated by a multilabel plate reader at an excitation wavelength of 530 nm and emission wavelength of 590 nm. Blank wells containing complete M199 medium were used to monitor the background fluorescence activity of resazurin, and the value was subtracted from every test well. Wells containing freely growing promastigotes in complete medium without inhibitors were used as positive controls (0% inhibition). Miltefosine, amphotericin B and pentamidine were incubated with promastigotes for comparison.

**Determination of 50% Inhibitory Concentration**

Median inhibitory concentration (IC₅₀) was estimated for compounds that demonstrated >70% inhibition at 1 µM during the primary screening. Two-fold serial dilutions (starting from 2 µM) in 100 µM of culture medium were made for each test concentration in triplicate. Parasite cultures containing 1×10⁶/mL logarithmic phase promastigotes were finally added to each well to achieve the maximum test concentration of 1 µM. The plates were then incubated for 68 h at 26°C in a 5% CO₂/air mixture. Activity of PB compounds was determined with the resazurin-based fluorescence assay measured after a total incubation time of 72 h using a multilabel plate reader at an excitation wavelength of 530 nm and emission wavelength of 590 nm. Promastigote inhibition (%) was calculated for each concentration using the following formula:

\[
\text{Inhibition(\%)} = \left( \frac{\text{Fluorescence in duplicate drug wells} - \text{average blank wells}}{\text{Average fluorescence control wells} - \text{average blank wells}} \right) \times 100
\]

All compounds were tested in two independent experiments and the DMSO concentration was kept below 1%.

**Hemolysis Test**

Hemolysis test was conducted according to a method described by Mahmoudvand et al²⁶ and Esteves et al.²⁷ In brief, 2% blood suspension was prepared in PBS (pH 7.2), from which 200 µL was taken and added to Eppendorf tubes containing test substances to achieve serially diluted concentrations starting from 25 µM. The blood suspension and the solution were then carefully mixed and incubated at 37°C for 2 h. Triton X-114 was used as a positive control (5 µL/mL) and incubated for 30 min. The mixture was centrifuged at 1,000 g for 10 min. Then, 75 µL of the...
resulting supernatant of each tube was transferred to 96-well plates and absorbance was measured at 540 nm. Blood suspension with 2.5% DMSO was used as a negative control.

**THP-1 Cell Cytotoxicity Assay**

The cytotoxic effect of selected compounds on THP-1 cell lines was assessed according to Habtemariam and Esteves et al., with minor modifications. In brief, precultured 50 µL suspensions of 1×10⁶ THP-1 cells were added to 96-well plates containing 50 µL serially diluted test molecules. The plates were then incubated for 68 h at 37°C in a 5% CO₂/air mixture. Cell viability was determined with the resazurin-based fluorescence assay measured after a total incubation time of 72 h using a multi-label plate reader at an excitation wavelength of 530 nm and emission wavelength of 590 nm. Cell viability (%), used to estimate median cell cytotoxicity (CC50), was calculated for each concentration using the following formula:

\[
\text{Cell viability} = \left( \frac{\text{Fluorescence in duplicate drug wells} - \text{average blank wells}}{\text{Average fluorescence control wells} - \text{average blank wells}} \right) \times 100
\]

**Selectivity Index**

The selectivity of the compound in killing pathogens as opposed to mammalian cells was assessed using the following formula:

\[
\text{Selectivity index (SI)} = \frac{\text{CC50 THP1 cells}}{\text{IC50 promastigotes or amastigote}}
\]

**Physicochemical Properties**

Physicochemical property data were collected from the National Centre for Biotechnology Information (NCBI). These data were used to check the suitability of test compounds for cell membrane penetration against Lipinski’s rule of absorption and permeation.

**Intracellular Amastigote Assay**

Peritoneal macrophages were counted using a hemocytometer and their total number was adjusted to 1×10⁶ cells/mL in complete MEM. Approximately 3×10⁵ macrophages were incubated in 24-well plates containing removable microscopic coverslips. Cells were allowed to adhere for at least 12 h at 37°C in 5% CO₂. Non-adherent cells were washed twice with prewarmed complete media and incubated overnight in fresh media. Adherent cells were then infected with late stationary stage *L. donovani* promastigotes with a parasite-to-cell ratio of 10:1 and incubated for a further 12 h. Non-internalized promastigotes were removed by extensive washing and plates were further incubated with or without test compounds and standards for 3 days at 37°C in a 5% CO₂ incubator. Amphotericin B and pentamidine were used as standards. After 72 h of incubation, slides were washed with PBS (prespurred at 37°C), fixed with methanol and stained with Giemsa (10%) for 15 min. Infection was judged to be adequate if more than 70% of the macrophages present in the untreated controls were infected. The IC₅₀ values of selected compounds against promastigotes were considered as the main criteria to select compounds for intracellular assay.

**Determination of IC₅₀ Against Intracellular Amastigotes**

The number of amastigotes was determined by counting amastigotes in at least 50 macrophages in duplicate cultures. The total actual parasite burden was calculated using the infection index, referred to as the associate index. The infection index for each well in duplicate was determined by multiplying the percentage of infected macrophages (IR) by the average number of amastigotes in infected cells, as shown below.²⁸⁻³⁰

\[
\text{Infection index} = \frac{\text{Number of infected macrophage}}{\text{total macrophage counted}} \times \frac{\text{total number of amastigotes}}{\text{in infected macrophages counted}}
\]

The IC₅₀ of each test compound was defined as the inhibitory concentration of the test compound that reduces the number of amastigotes per infected macrophage by 50%.

**Evaluation of Synergistic Activity of Molecules**

In vitro drug interactions were assessed using the combination index method as described by Chou and Martin.³¹ The dose–response relationship of each drug alone and in combination was assessed separately. Drug combinations were made using IC₅₀ values of individual drugs. Two data point serial dilutions were made above and below the midpoint to determine the highest and lowest concentrations. Each drug was then mixed at each respective IC₅₀ (5:5, 4:4, 3:3, 2:2, 1:1), where 3:3 is the median inhibitory concentration of the two drugs. The combination index (CI) values were estimated to be CI <1 =1 and >1, which indicate the presence of synergism, additive effect and antagonism, respectively.
Statistical Analysis
Percentage inhibition for test and reference compound was expressed as mean value ± standard deviation. The IC$_{50}$ for promastigotes and intracellular amastigotes and the CC$_{50}$ for the cytotoxicity assay on the THP-1 cells were evaluated by non-linear regression analysis using GraphPad Prism version 7.00 (GraphPad Software, 2016), each expressed as mean ± 95% CI from two independent experiments, with each test concentration tested in triplicate. The drug combination effect of selected compounds with the reference drugs was assessed using isobolograms, and analysis was carried out using CompuSyn (CompuSyn 1.0; ComboSyn, 2005).

Ethics Statement
All procedures performed, including the use of THP-1 cells, were reviewed and approved by the Departmental Research Ethics Committee, Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, College of Health Sciences, Addis Ababa University. Animal handling procedures and management of protocols were carried out in accordance with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health.

Results and Discussion
Primary Screening Results
Primary screening of the library consisting of 400 compounds against *L. donovani* was conducted to evaluate activity against the promastigote stage of *L. donovani* at 1 μM. After the primary screening, the average percentage inhibition was determined and a heat map was generated for the five 96-well PB plates. The map was developed using the average percentage of inhibition of each compound obtained from two independent experiments in triplicate. Then compounds were classified under different ranges of percentage inhibition (Supplementary Figure S01). From the total of 400 compounds screened, 35 compounds showed >50% inhibitory activity on promastigotes in the initial screen (1 μM). Out of these compounds, 11 showed >70% inhibition. From the 11 compounds, two compounds (Plate A well D06 and Plate B well A04) were purposively omitted from further investigation. Plate A well D06 (MMV688942), despite its considerable activity on the primary screening, failed to demonstrate a dose-dependent inhibitory effect in the subsequent dose titration assay, whereas plate B well A04 (pentamidine) is a standard drug for leishmaniasis (MMV000062).

Active compounds which demonstrated above 70% inhibition were selected for further study (Table 1). MMV688942, despite its considerable activity on the primary screening, failed to demonstrate a dose-dependent inhibitory effect in the subsequent dose titration assay. The other compound, B-A04 (MMV000062), was pentamidine, which is a standard drug for leishmaniasis treatment.

Most compounds identified in the present study were obtained from disease sets which share common features with the *Leishmania* parasite. Two compounds (MMV022478, MMV011511) were obtained from the malaria disease set, three (MMV690102, MMV688415 and MMV690103) from the kinetoplastid disease set, one compound from the tuberculosis disease set (MMV688262), and the rest (MMV675968, MMV688703 and MMV637229) were obtained from the cryptosporidiosis, toxoplasmosis and trichuriasis disease sets, respectively.

Pyrimido[4,5-d]pyrimidine-2,4,7-triamine chemotype derivatives MMV675968, MMV690102 and MMV690103 are known to inhibit dihydrofolate reductase (DHFR). Anti-leishmanial activity of MMV690102 and MMV690103 was previously reported, which strengthens the assumption of leishmanial DHFR being a target for these compounds. MMV637229 (clemastine) and MMV688703 were reported for their activity on

| Compound | Plate-Well Location | MMV ID    | Molecular Weight | Molecular Formula |
|----------|---------------------|-----------|------------------|-------------------|
| 1        | B-F5                | MMV637229 | 459.97           | C$_{21}$H$_{26}$NOCl |
| 2        | C-D8                | MMV690103 | 349.43           | C$_{16}$H$_{16}$N$_7$ |
| 3        | C-E8                | MMV690102 | 417.46           | C$_{21}$H$_{22}$N$_7$O$_3$ |
| 4        | C-F6                | MMV688262 | 534.48           | C$_{23}$H$_{24}$N$_7$O$_3$F$_3$ |
| 5        | C-G11               | MMV688703 | 335.42           | C$_{21}$H$_{28}$N$_7$F |
| 6        | D-B2                | MMV011511 | 367.56           | C$_{22}$H$_{27}$N$_7$O |
| 7        | D-H3                | MMV022478 | 545.93           | C$_{23}$H$_{29}$N$_7$OCl |
| 8        | D-H9                | MMV675968 | 359.81           | C$_{21}$H$_{28}$N$_7$O$_3$Cl |
| 9        | E-G11               | MMV688415 | 433.54           | C$_{23}$H$_{29}$N$_7$O$_3$ |
histamine and cGMP-dependent kinase, respectively. MMV022478 is a member of the pyrazolo[1,5-a]pyrimidine class, and this class has been identified as an inhibitor of mammalian NADPH oxidase-4. Hence, it is more likely to inhibit *Leishmania* trypanothione reductase. The activity of MMV688415 on *Trypanosoma cruzi* was reported previously, without convincing evidence on its biological target. However, MMV688262 (delamanid) has been extensively studied for its anti-leishmanial activity. This compound is a dihydro-nitroimidazo-oxazole derivative, acting by inhibiting the synthesis of mycobacterial cell-wall components, methoxymycolic acid and ketomycolic acid. It is likely that this compound also targets *Leishmania* cell-wall components. The remaining compound (MMV011511) does not have a reported mode of action. The molecular structures of the nine compounds which reduced promastigote viability to less than 30% are shown in Figure 1.

The IC\textsubscript{50} of anti-promastigote activity was estimated for compounds which demonstrated strong inhibitory activity at 1 \( \mu \text{M} \). The IC\textsubscript{50} values for all tested compounds were below 0.5 \( \mu \text{M} \); of these, the DHFR inhibitor (MMV690102) demonstrated very low IC\textsubscript{50} (0.012 and 0.055 \( \mu \text{M} \)) in two independent experiments (Table 2). This finding is consistent.

**Figure 1** Structures of selected Pathogen Box compounds with >70% promastigote growth inhibition.
with previous reports on the activity of MMV690102 against *Leishmania mexicana* and the activity of MMV690102 against *L. donovani* strains. The IC$_{50}$ of MMV688262 in the current study was slightly higher than in the study by Patterson et al, who reported 0.015 μM L. donovani promastigotes. This may have resulted from the differences in assay conditions or strains, and the amount of serum used.

### Cytotoxicity Assay

The safety of selected compounds was assessed on sheep red blood cells and human monocytes (THP-1 cells) in a two-fold serial dilution of test compounds (25–0.05 μM). All compounds, except for one (MMV675968 = 6.9% hemolysis), had less than 5% hemolysis at 25 μM, demonstrating the minimum risk of hemolytic activity at therapeutic concentrations. MMV690102 (in the kinetoplastid disease set of the PB) demonstrated activity on THP-1 cells, with an extrapolated CC$_{50}$ value of 36.55 μM (95% CI: 29.86–45.36). The estimated SI of MMV690102 with respect to THP-1 cells was found to be 664.54, which is greater than the 100-fold selectivity window set for *Leishmania* parasites. This compound also displayed no activity in the HEK293 assay performed by Duffy et al. However, biological activity data provided by MMV showed that MMV690102 demonstrated significant inhibitory activity on MRC-5 and PMM cells at 5.4 μM and 4 μM, respectively. These variations in the CC$_{50}$ of test compounds for different cells reflect the need for further studies to outline the effect of this compound on many other types of mammalian cells.

### Assessment of Physicochemical Properties of Selected Compounds

Physicochemical assessment was conducted to validate the compounds' drug likeness and suitability for intracellular amastigote assays. Relevant physicochemical data were gathered from the NCBI and a reference table was constructed and weighed against Lipinski's rule of absorption and permeation.

Target compounds for intracellular amastigote assays should have good cell permeability and absorption to enable the compound to traverse through two membranes and reach the site of action. Lipinski's rule of five (RO5) indicates that compounds which have more than 5 H-bond donors, 10 H-bond acceptors, molecular weight greater than 500 and computational log P (ClogP) greater than 5 (or Moriguchi log P [MlogP] >4.15) are associated with poor absorption and/or permeation. Table 3 shows the physicochemical properties of the selected hits, labeled as R1–R6. According to the physicochemical analysis, the antitubercular agent (anti-TB agent) MMV688262 (delamanid) has a molecular weight of 534.4 g/mol and hydrogen receiver

### Table 2 Activity of Selected Compounds Against *Leishmania donovani* Promastigotes

| Rank | MMV ID        | Experiment 1 |                | Experiment 2 |                | Average IC$_{50}$ |
|------|---------------|--------------|----------------|--------------|----------------|-------------------|
|      |               | IC$_{50}$ (95% CI) (µM) | R$^2$ | IC$_{50}$ (95% CI) (µM) | R$^2$ | IC$_{50}$ (95% CI) (µM) | R$^2$ | IC$_{50}$ (95% CI) (µM) | R$^2$ |
| 1    | MMV690102     | 0.012 (0.002–0.022) | 0.7435 | 0.055 (0.030–0.078) | 0.794 | 0.033 |
| 2    | MMV688415     | 0.224 (0.129–0.369) | 0.6237 | 0.162 (0.117–0.214) | 0.8697 | 0.193 |
| 3    | MMV022478     | 0.295 (0.213–0.401) | 0.7944 | 0.124 (0.042–0.213) | 0.5551 | 0.209 |
| 4    | MMV688262     | 0.233 (0.192–0.291) | 0.9001 | 0.262 (0.204–0.338) | 0.8895 | 0.249 |
| 5    | MMV637229     | 0.371 (0.266–0.551) | 0.7689 | 0.141 (0.110–0.175) | 0.9044 | 0.256 |
| 6    | MMV011511     | 0.188 (0.127–0.261) | 0.769 | 0.337 (0.247–0.474) | 0.7913 | 0.262 |
| 7    | MMV688703     | 0.175 (0.035–0.221) | 0.873 | 0.361 (0.289–0.458) | 0.8665 | 0.268 |
| 8    | MMV675968     | 0.295 (0.217–0.412) | 0.8174 | 0.299 (0.219–0.420) | 0.814 | 0.297 |
| 9    | MMV690103     | 0.491 (0.353–0.767) | 0.7567 | 0.168 (0.109–0.241) | 0.739 | 0.329 |

**Abbreviations:** 95% CI, 95% confidence interval; IC$_{50}$, median inhibitory concentration; R$^2$, regression coefficient.
count of 11, both of which are slightly above the limits set by Lipinski RO5, whereas the remaining compounds comply with Lipinski’s rules of permeation and absorption. The molecular weight and hydrogen bond count often improve the affinity and selectivity of drugs. Hence, it is difficult for some drugs to achieve RO5 compliance. This may be the case for delamanid, which failed to meet the RO5.

The objective of this screening was to identify compounds with activity in the submicromolar range which qualified for Lipinski’s rules. In doing so, compounds that showed minimum IC\(_{50}\) (average of two independent experiments) from the anti-promastigote assays were selected for intracellular amastigote assays. These included MMV022478, MMV688262, MMV688415 and MMV690102. MMV688262, despite its violation of RO5 compliance, was included in the intracellular amastigote assay because of previous research reports.

### Activity of Selected Compounds Against L. donovani Amastigotes

The selected nine compounds were screened at 1 \(\mu\)M for their activity against intracellular amastigotes. Among which, only four compounds showed >50% inhibition. The IC\(_{50}\) value of the four tested compounds was then assessed. All tested compounds showed concentration-dependent inhibition against intracellular amastigotes of L. donovani. Among these compounds, MMV690102 and MMV688262 (delamanid) were found to be potent inhibitors, with IC\(_{50}\) values of 0.06 and 0.053 \(\mu\)M, respectively. These values are comparable to the IC\(_{50}\) of amphotericin B (0.076 \(\mu\)M) observed in this assay (Table 4).

The current finding on MMV688262 (delamanid) is within the range of a report by Patterson et al.,\(^{38}\) who investigated the activity of delamanid against various clinical isolates of L. donovani with IC\(_{50}\) values from 86.5 to 259 \(\mu\)M. The lowest IC\(_{50}\) (86.5 \(\mu\)M) was registered on L. donovani isolates obtained from Ethiopia, which substantiate our report. Berry et al reported an IC\(_{50}\) value of 1.780 \(\mu\)M on L. mexicana. This variance may be caused by differences in the assay format or species variation.\(^{28}\) Delamanid is a pro-drug, which belongs to dihydronitroimidazole class, developed to be an orally active drug for the treatment of TB.\(^{45}\) The drug has gone through advanced clinical assessment and has already been approved by many regions for the treatment of susceptible TB\(^{46}\) and multi-drug-resistant TB. It was proven to be rapidly bactericidal and capable of shortening treatment duration.\(^{45}\)

A study on different L. donovani strains revealed the potential use of delamanid in the treatment of leishmaniasis.\(^{38}\) Delamanid demonstrated a rapid leishmanicidal activity, good safety profile, good solubility in aqueous solution, a high volume of distribution and very small renal excretion (<5%), making it suitable for patients with renal impairment and mild hepatic insufficiency.\(^{39-41,47}\) It is suitable for oral administration, which makes it the second orally active drug, following miltefosine, for the treatment of leishmaniasis. Anti-leishmanial activity of delamanid is particularly important for HIV co-infected patients, as studies show that delamanid administered with anti-retroviral and anti-TB drugs (tenofovir, efavirenz, ritonavir and rifampin) shows no clinically relevant interaction.\(^{45,48}\) The mode of action of delamanid was shown to inhibit synthesis of the mycobacterial cell-wall components, methoxymycolic and ketomycolic acid.\(^{45,49}\) Although different scholars assumed that the drug is biologically activated by bacterial-like nitroreductase (NTR),\(^{38,39,50}\) Patterson et al showed that NTR is not responsible for the activation of delamanid,\(^{38}\) clearly implying that a different mechanism of action is at play.

Anti-leishmanial activity of MMV690102 was reported by Berry et al\(^{28}\) and Duffy et al.\(^{32}\) Both studies identified

| Plate-Well Location | MMV ID  | IC\(_{50}\) (95% CI) (\(\mu\)M) | \(R^2\) |
|--------------------|--------|-----------------------------|-------|
| C-F6               | MMV688262 | 0.053 (0.044–0.061)       | 0.9791|
| C-E8               | MMV690102 | 0.060 (0.047–0.072)       | 0.9618|
| E-G11              | MMV688415 | 0.704 (0.530–0.858)       | 0.9523|
| D-H3               | MMV686478 | 0.694 (0.560–0.822)       | 0.9708|

| Amphotericin B     | 0.076 (0.051–0.092) |
| Pentamidine        | 1.83 (0.662–2.99)   |

Abbreviations: 95% CI, 95% confidence interval; IC\(_{50}\), median inhibitory concentration; \(R^2\), regression coefficient.

### Table 3 Relevant Physicochemical Information of Selected Compounds

| S.N. | MMV ID | R1     | R2     | R3  | R4  | R5  | R6     | Source |
|------|--------|--------|--------|-----|-----|-----|--------|--------|
| 1    | MMV011511 | 367.5  | 4      | 4   | 8   | 3.9 | 27.7   | [45]   |
| 2    | MMV022478 | 432.9  | 2      | 5   | 4   | 3.2 | 74.6   | [46]   |
| 3    | MMV637229 | 343.9  | 0      | 2   | 6   | 5   | 12.5   | [47]   |
| 4    | MMV675968 | 359.1  | 3      | 7   | 5   | 2.9 | 108    | [48]   |
| 5    | MMV688262 | 534.4  | 0      | 11  | 7   | 5.6 | 104    | [49]   |
| 6    | MMV688415 | 433.5  | 2      | 6   | 6   | 2.3 | 69.1   | [50]   |
| 7    | MMV688703 | 335.1  | 1      | 3   | 3   | 3.6 | 31.9   | [51]   |
| 8    | MMV690102 | 417.4  | 2      | 9   | 6   | 3.3 | 125    | [25]   |
| 9    | MMV690103 | 349.4  | 2      | 7   | 3   | 3.2 | 107    | [3.5]  |

Notes: R1, molecular weight (g/mol); R2, \(H^+\) bond donor; R3, \(H^+\) bond receiver; R4, rotatable bonds; R5, XlogP3; R6, polar surface area (A\(^2\)).
MMV690102 as a promising starting point for further drug development. MMV690102 was believed to be a DHFR inhibitor. However, Duffy et al., showed that MMV690102 analogues are ineffective against *P. falciparum* and *T. brucei*. There is no logical explanation as to why this compound failed to act, as both parasites are known to be inhibited by DHFR inhibitors, unless it can be explained by the structural divergence between DHFR orthologues expressed in many organisms.

Reports of anti-leishmanial activity by the remaining compounds are limited, except for the report by MMV on MMV688415 activity against *L. infantum* amastigotes (IC$_{50}$ = 17.3 μM) (MMV biological activity data).

**Identified Leads for Optimization**

Screening of the PB compounds identified more than nine potential hits suitable for “hit to lead” optimization against *L. donovani* infection, of which the activity of four compounds (MMV688415, MMV022478, MMV690102 and MMV688262) against intracellular amastigotes was confirmed by the present study. We could not suggest MMV688415 and MMV022478 as potential leads this time, as there are not enough biological activity data. Based on the findings in the present study, and taking evidence from other studies into account, as well as data on pharmacokinetic profiles, MMV690102 and MMV688262 are potential lead compounds for further drug development.

**Synergistic Effect of Lead Compounds with Reference Drugs**

Drug resistance and treatment failures have been reported from various regions of Africa, India and Asia. As a result, there is a need for new anti-leishmanial drugs or combination therapy to treat drug-resistant leishmanial infections. With the goal of identifying effective drug combinations, we investigated the synergistic effects of MMV690102 and MMV688262 with amphotericin B. The two combinations were tested in the ratio of 1:1. Isobologram analysis for the combination of amphotericin B and delamanid showed that their combination index is less than 1 at an inhibitory relevant concentration to affect the parasite (Figure 2). This indicates that the two compounds have synergistic activity. The synergistic activity was not, however, observed from the combination of MMV690102 and amphotericin B.

The synergistic effect of delamanid combination with amphotericin B to inhibit the intracellular amastigote stages of *L. donovani* shows the potential of this combined regimen in the treatment of resistant leishmaniasis. Further in vitro and in vivo studies are, however, required in resistant and sensitive strains to gain an insight into its potential in the development of a combination treatment for drug-resistant leishmaniasis.

**Conclusion**

The present study identified hit compounds that have desirable inhibitory activity against promastigotes and amastigotes of *L. donovani* patient isolates, with additional data that suggest the identified compounds to be safe. However, in vitro drug screening efficacy and safety do not necessarily reflect the in vivo situation. Hence, this study should be complemented with more in vivo studies. From the identified hits, only four compounds were investigated in the intracellular amastigotes assay based on activity against promastigotes. The activity of the remaining compounds needs to be further investigated. The two lead compounds (MMV690102 and MMV688262) identified in this study should be taken forward for further in vivo and other preclinical studies. Additional target-based experiments are also expected to provide further insights into the synergistic effects of these compounds with reference drugs.
needed to understand their anti-leishmanial mechanisms of action.

**Data Sharing Statement**
Data will be available upon request.

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**Author Contributions**
All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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**Disclosure**
The authors declare that they have no competing interests in this work.

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