Identification of Small Molecule Lead Compounds for Visceral Leishmaniasis Using a Novel Ex Vivo Splenic Explant Model System

Yaneth Osorio1,²*, Bruno L. Travi1,²*, Adam R. Renslo3, Alex G. Peniche1,²*, Peter C. Melby1,²,4*  

1 Department of Veterans Affairs Medical Center, Research Service, South Texas Veterans Health Care System, San Antonio, Texas, United States of America, 2 Department of Medicine, The University of Texas Health Science Center, San Antonio, Texas, United States of America, 3 Small Molecule Discovery Center, Sandler Center for Basic Research in Parasitic Diseases, and Department of Pharmaceutical Chemistry, University of California San Francisco, San Francisco, California, United States of America, 4 Department of Microbiology and Immunology, The University of Texas Health Science Center, San Antonio, Texas, United States of America

Abstract

Background: New drugs are needed to treat visceral leishmaniasis (VL) because the current therapies are toxic, expensive, and parasite resistance may weaken drug efficacy. We established a novel ex vivo splenic explant culture system from hamsters infected with luciferase-transfected Leishmania donovani to screen chemical compounds for anti-leishmanial activity.

Methodology/Principal Findings: This model has advantages over in vitro systems in that it: 1) includes the whole cellular population involved in the host-parasite interaction; 2) is initiated at a stage of infection when the immunosuppressive mechanisms that lead to progressive VL are evident; 3) involves the intracellular form of Leishmania; 4) supports parasite replication that can be easily quantified by detection of parasite-expressed luciferase; 5) is adaptable to a high-throughput screening format; and 6) can be used to identify compounds that have both direct and indirect anti-parasitic activity. The assay showed excellent discrimination between positive (amphotericin B) and negative (vehicle) controls with a Z’ Factor >0.8. A duplicate screen of 4 chemical libraries containing 4,035 compounds identified 202 hits (5.0%) with a Z Score of < -1.96 (p<0.05). Eighty-four (2.1%) of the hits were classified as lead compounds based on the in vitro therapeutic index (ratio of the compound concentration causing 50% cytotoxicity in the HepG2 cell line to the concentration that caused 50% reduction in the parasite load). Sixty-nine (82%) of the lead compounds were previously unknown to have anti-leishmanial activity. The most frequently identified lead compounds were classified as quinoline-containing compounds (14%), alkaloids (10%), aromatics (11%), terpenes (8%), phenothiazines (7%) and furans (5%).

Conclusions/Significance: The ex vivo splenic explant model provides a powerful approach to identify new compounds active against L. donovani within the pathophysiologic environment of the infected spleen. Further in vivo evaluation and chemical optimization of these lead compounds may generate new candidates for preclinical studies of treatment for VL.

Introduction

New drugs are desperately needed to treat visceral leishmaniasis (VL), and this in turn requires new approaches to discover novel lead compounds that might populate a pipeline of new therapeutics for patients with VL. Current therapies for the leishmaniases are toxic, difficult to deliver, expensive, and their efficacy is hindered by parasite resistance (reviewed in [1]). The pentavalent antimony compounds, sodium stibogluconate and meglumine antimoniate, have been the mainstay of anti-leishmanial chemotherapy for more than 40 years. The recommended regimen involves prolonged and often repeated courses of drug administered by the intravenous or intramuscular routes. Cure rates of 80–100% were common in the 1990s, but have dropped off considerably because of parasite resistance [2]. Adverse effects of antimony therapy are multiple and often dose-limiting. Amphotericin B desoxycholate and the amphotericin lipid formulations are also used in the treatment of VL, and in many regions have replaced antimony as first-line therapy. The use of these drugs, however, is limited by their difficulty of administration, well-known risk of toxicity, and high cost. Parenteral treatment of VL with the aminoglycoside paromomycin (aminosidine) is used in India but not licensed in the U.S. Miltefosine, a membrane targeting alkylphospholipid, was recently licensed in India as the first oral treatment for VL, but after only a few years of use, drug resistance has emerged. The discovery of...
Visceral leishmaniasis is a life threatening parasitic disease present in several countries of the world. New drugs are needed to treat this disease because treatments are becoming increasingly ineffective. We established a novel system to screen for new anti-leishmanial compounds that utilizes spleen cells from hamsters infected with the parasite Leishmania donovani. The parasite strain we used was genetically engineered to emit light by the incorporation of the firefly luciferase gene. This laboratory test system has the advantage of reproducing the cellular environment where the drug has to combat the infection. The efficacy of the compounds is easily determined by measuring the light emitted by the surviving parasites in a luminometer after exposing the infected cells to the test compounds. The screening of more than 4,000 molecules showed that 84 (2.1%) of them showed anti-leishmanial activity and had an acceptable toxicity evaluation. Eighty-two percent of these molecules, which had varied chemical structures, were previously unknown to have anti-leishmanial activity. Further studies in animals of these new chemical entities may identify drug candidates for the treatment of visceral leishmaniasis.

Visceral leishmaniasis is a life threatening parasitic disease present in several countries of the world. New drugs are needed to treat this disease because treatments are becoming increasingly ineffective. We established a novel system to screen for new anti-leishmanial compounds that utilizes spleen cells from hamsters infected with the parasite Leishmania donovani. The parasite strain we used was genetically engineered to emit light by the incorporation of the firefly luciferase gene. This laboratory test system has the advantage of reproducing the cellular environment where the drug has to combat the infection. The efficacy of the compounds is easily determined by measuring the light emitted by the surviving parasites in a luminometer after exposing the infected cells to the test compounds. The screening of more than 4,000 molecules showed that 84 (2.1%) of them showed anti-leishmanial activity and had an acceptable toxicity evaluation. Eighty-two percent of these molecules, which had varied chemical structures, were previously unknown to have anti-leishmanial activity. Further studies in animals of these new chemical entities may identify drug candidates for the treatment of visceral leishmaniasis.

Materials and Methods

Animals and parasites

Female inbred Chester Beatty hamsters (6–8 weeks-old) derived from our own breeding colony were used. Leishmania donovani (MHOM/SD/001S-2D) promastigotes were cultured in complete M199 (0.12 mM adenine, 0.0005% Hemin, 20% FBS) as described previously [18]. The L. donovani strain was transfected with an episomal vector containing the luciferase (luc) reporter gene [19] and was maintained routinely by isolation from infected hamsters, selection in complete M199 with 10 µg/ml of G418, and intracardial subinoculation of new hamsters approximately every 3 months. These studies were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center.

Development of the ex vivo splenic explant culture

Groups of 8 animals were infected by the intracardial route with 10⁷ purified metacyclic promastigotes [11] and the body and spleen weights recorded at 7, 14 and 21 days post-infection. At each time point, the splenocytes were harvested as described below and the number of cells and parasite burden determined by microscopy and luminometry, respectively. The luminometry counts were transformed to number of parasites using a linear standard curve of luciferase activity versus number of microscopically enumerated amastigotes. The amastigotes used for the standard curve were isolated from a cell suspension from hamster spleen at 20–30 days p.i. as follows: A splenocyte suspension was prepared by passing the spleen through a wire mesh and the splenocytes were disrupted by passing sequentially through 27G and 30G½ needles, and polycarbonate membrane filters having pore sizes of 8 µm, 5 µm and 3 µm (Isoapore, Millipore). Released amastigotes (free of host cells) were washed twice with PBS (3000 g × 10 min) and enumerated by direct microscopy. The alternative activation of macrophages was assessed by determining the arginase activity by the rate of urea formation from L-arginine in the presence of 1-phenyl-1,2-propanedione-2-oxide (ISPF) [20] and measuring the soluble collagen content (Sircol Assay, Bicolor Ltd). The cellular immune response was evaluated by the proliferative response of spleen cells to Concanaavalin A as described [21].
Cell populations in the ex vivo spleen explant culture

The general cell composition of the spleen explant was determined by microscopy and flow cytometry at 7, 14 and 21 days post-infection and compared with uninfected spleen cells. The spleen cells were suspended in DMEM +5% FBS at 10^5 cells/100 μL, washed with PBS plus 0.1% BSA and 0.025% sodium azide, blocked for 20 min with PBS with 2% BSA and 5% of normal serum of the species in which the secondary antibodies were raised, and labeled with the monoclonal antibodies that are known to cross react with the corresponding hamster molecules. CD4 T cells were quantified by staining with rat anti-mouse CD4-PE (clone GK1.5; BD, 0.5 μg/tube) [22] followed by fixation, permeabilization (Leucoparam; Serotec), and labeling with rat anti-human CD3-FTTC (CD3-12; Serotec, 0.5 μg per tube) which recognizes a highly conserved intracellular epitope of the CD3 molecule expressed by T lymphocytes in several species (manufacturer’s data). B lymphocytes were quantified by labeling cells that expressed both the MHCII alloantigen (mouse anti-mouse I-EK)-PE (clone 14-4-18S, BD, 0.5 μg/tube) [23] and IgG (Goat F(ab’2) anti-hamster IgG (clone Dlight 488; Serotec, 1 μg per tube). In all cases the percent of positive cells was determined by flow cytometry (FacsAria, BD) according to the threshold of the corresponding isotype controls.

Parasite replication in the ex vivo spleen explant culture

The number of parasites and the proportion of infected macrophages were determined after 0, 24, 48 and 72 hours of ex vivo culture at 37°C, 5% CO2 using luminometry and light microscopy. The ability of the lumimetric assay to discriminate between treated and untreated (control) splenic explant cultures was assessed in serial two-fold dilutions of cultures with 48 h in 100 μL of 0.2 μM amphotericin B (Sigma) or vehicle control.

Splenic explant culture for drug screening and EC50 determinations

To establish the plate assay for drug screening, the spleens from 2 infected hamsters were aseptically removed and placed in a Petri dish containing 5 mL of Collagenase D (Roche) at 2 mg/mL of buffer (10 mM Hepes pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2). The spleen was infiltrated by injection with approximately 2 mL of Collagenase solution, the tissue was cut into small pieces using sharp scissors, and incubated for 20 minutes at 37°C. The cell suspension and remaining tissue fragments were gently passed through a 100 μm strainer (B-D) to obtain a single cell suspension, which was washed twice by centrifugation (500 g for 7 min at 4°C) and re-suspended in culture medium composed of DMEM (Gibco), 5% FBS, 1 mM Sodium pyruvate (Gibco), 1X MEM amino acids solution (Sigma), 0.1% w/v/EDTA, 10 mM HEPES buffer (Gibco) and 100 μg/mL streptomycin solution (Cellgro). The splenocytes were counted and adjusted at concentrations from 100,000 to 500,000 cells/50 μL and used the same day for drug screening or determination of EC50 as described below.

Primary screening of compound libraries using the ex vivo splenic explant model

The following chemical libraries in 96-well plate format were screened: NINDS Custom Collection II (MicroSource Discovery Systems, Inc.), which consists of 1,040 classical therapeutic agents, established experimental inhibitors, receptor agonist drugs and other bioactive compounds [24,25,26]; the Pure Natural Products library (MicroSource Discovery Systems, Inc.) a collection of 800 pure natural products and derivatives; and The Diversity Set and Natural Products set (Developmental Therapeutics Program, NCI/NHI) a set of 2,195 compounds selected from the almost 140,000 compounds based on diversity of structure [27,28,29]. Libraries containing the compounds at 10 nM concentration in DMSO were diluted into master plates at 200 μM concentration in DMEM. The 200 μM master plates were further diluted by transferring an aliquot of 5 μl into a 96-well sterile white-bottom plate (Costar) containing 45 μl of culture medium at 4°C. Splenocytes (100,000 in 50 μl of culture medium) that had been obtained from infected hamsters as described above were added to the assay plate for a final drug concentration of 10 μM. The positive control (splenocytes treated with 0.2 μM Amphotericin B) and negative controls (0.1% DMSO vehicle) were distributed in the two outer columns (8 wells each) of each plate in alternating fashion to minimize any edge effect [30]. After 48 hours of culture at 37°C in a humidified atmosphere and 5% CO2, the plates were centrifuged at 500 g for 7 min, the supernatant discarded using a multi-channel vacuum aspirator (Costar), and 20 μl of 1X cell culture lysis reagent (Promega) was added to the cells. To complete the lysis procedure the plates were frozen at ~70°C and then thawed, and the luciferase activity determined in a plate luminometer following addition of 100 μl of the luciferin substrate at room temperature (Promega). The Z score was calculated for each compound as the mean counts of the compound tested minus the mean counts of all compounds in the plate divided by the SD all compounds in the plate [30]. The mean Z score for each drug was calculated based on duplicate screenings performed in two different experiments. A Z score of ≤–1.96, which corresponds to a p value of ≤0.05 (95% confidence limit), was used as the threshold to identify the hits [30]. The conditions of the screening were optimized to obtain the best signal to noise ratio by calculating the Z' factor obtained after exposure to different drug concentrations (10, 5, 2.5 μM). The quality of each assay was determined by calculating the Z' prime (Z') factor [31], which measures the discrimination between positive and negative controls in the screen. The Z' factor was calculated as 1 − [3SD positive controls +3SD negative controls/absolute value of (mean of the positive controls – mean of the negative controls)].

Assessment of cell toxicity (CC50) using the HepG2 cell line

We used a cell-based assay as an alternative to animal testing to determine the toxicity of the identified hit compounds [32]. The cytotoxicity was evaluated in HepG2 cells (human hepatocellular carcinoma, ATCC#HB 8065) maintained in MEM (Gibco) supplemented with 10% FBS 1 mM sodium pyruvate (Gibco), 1X MEM aminocids solution (Sigma), 0.1% w/v/EDTA, 10 mM HEPES buffer (Gibco) and 100 μg/mL streptomycin solution (Cellgro). The HepG2 cell monolayers were detached using 1X trypsin/EDTA (Gibco), washed and adjusted to 500,000 cells/mL in supplemented MEM, and 50 μl of the cell suspension were added to white-bottom 96-well plates (25,000 per well) containing 50 μl of serial 2-fold dilutions of the test compounds (0.1–100 μM) or the DMSO control. After 24 hours of culture at 37°C the number of viable cells was determined by quantification of the ATP present in the cell using the CellTiter-Glo luminescent Cell Viability Assay (Promega). The luminescence values were used to construct a curve using a linear regression model (GraphPad Prism 5.0) and calculate the cytotoxic concentration that killed 50% of the cells (CC50). The mean and standard error of 3–5 different experiments were considered as the final CC50 for the purpose of calculating the in vitro therapeutic index (see below).
Anti-leishmanial efficacy (EC$_{50}$) using the ex vivo splenic explant model

The anti-leishmanial efficacy of the compounds was determined using the splenic explant model in a 96-well plate format. In brief, splenocytes from infected hamsters were obtained as described above and a suspension of 100,000–500,000 in 50 µL of culture medium were added to white-bottom 96-well plates containing 50 µL of serial 2-fold dilutions of test compounds (0.03–20 µM) in culture medium. Because variation in the parasite burden between different explant cultures was expected, the concentration of cells used in each experiment was adjusted to give approximately 100 counts by luminometry (equivalent to ~240,000 parasites). The number of surviving parasites was determined by luminometry as described above. Luminometry values were used to construct a curve using a linear regression model (GraphPad Prism 5.0) and calculate the effective concentration of the compound that killed 50% of the parasites (EC$_{50}$). The mean and standard error of the EC$_{50}$ from 2–3 different experiments were considered as the final EC$_{50}$ for the purpose of ranking the compounds and calculating the in vitro therapeutic index (see below).

Anti-leishmanial efficacy (EC$_{50}$) using the in vitro infected macrophage model

Macrophages from uninfected hamsters were obtained by peritoneal lavage with DMEM (Gibco) and Heparin (2 units/ml; Elkins-Sinn, Cherry Hill NJ). The cells were washed twice by centrifugation and re-suspended in culture medium composed of DMEM, 5% FBS, 1 mM sodium pyruvate (Gibco), 1X MEM amino acids solution (Sigma), 0.02% v/v/EDTA and 10 mM HEPES buffer (Cellgro). The peritoneal macrophages were adjusted to 5×10$^5$ cells/ml and allowed to adhere overnight at 37°C and 5% CO2 in flat bottom 96-well plates. Adherent macrophages were infected at 1:5 ratio (cells:parasites) with stationary phase LUC-transfected $L. donovani$ promastigotes and cultured at 37°C, 5% CO2, for 2 h. The extracellular parasites were then removed by washing with warm Dulbecco’s PBS.

Figure 1. Rationale for selection of the time for establishing the ex vivo splenic explant culture. Hamsters infected with 10$^6$ Luc-transfected $L. donovani$ were evaluated from 7 to 21 days post infection (n = 6 per time point). (A) Spleen weight. Shown is the mean ± standard deviation (SD) of the spleen to body weight ratio (spleen weight divided the body weight). (B) Splenic parasite burden. The number of amastigotes (mean ± SD) was determined by luminometry in 500,000 splenocytes by extrapolating the counts (photons/sec) to a standard curve of microscopy-enumerated spleen-derived amastigotes. (C) Total splenocyte number. Splenocyte number (mean ± SD) was determined by counting the cells by microscopy. (D) Lymphoproliferative response. The splenocyte stimulation index (shown as the mean ± SD) was determined by dividing the cpm of concanavalin A-stimulated and non-stimulated splenocytes. (E) Splenic soluble collagen content. The soluble collagen content (shown as the mean ± SD) was determined in spleens from uninfected and infected hamsters by the Sircol assay (Biocolor). (F) Splenic Arginase activity. Tissue arginase activity was determined by measurement of urea catalysis and is shown as the mean ± SD. Statistical analysis for all panels was performed by one-way analysis of variance (ANOVA).

doi:10.1371/journal.pntd.0000962.g001
Identification of lead compounds

Hit compounds that showed a significant Z score in the ex vivo screening but demonstrated high toxicity for the HepG2 cell line (CC50 ≤ 10 μM) were excluded from further consideration. After the exclusion of these toxic compounds, the in vitro therapeutic index (IVTI), which is the ratio of the CC50 obtained in the HepG2 cell line and the anti-leishmanial activity (EC50), was calculated for each of the hit compounds. The resulting IVTI was used to rank the hits and select lead compounds that had an IVTI > 5.

Results

Characterization of the ex vivo explant culture

We first characterized the clinical, immunological, and parasitological features of the infected hamster spleen early in the course of VL (which is ultimately fatal) to identify a time point that indicated the course of infection was transitioning toward severe disease. At 21 days p.i. there was a dramatic increase in spleen size (Fig. 1A), parasite burden (Fig 1B), and cellular infiltration of the spleen (Fig 1C). The splenomegaly was related to hypercellularity with a significant expansion of the macrophage population (6-fold increase over uninfected spleens, p = 0.016) and B lymphocytes (Table 1). The expansion of other cell populations that were not enumerated, such as fibroblasts, is likely to also contribute to the splenomegaly. The transition to progressive disease was also accompanied by loss of T cell responsiveness (Fig 1D) and preceded by a transient decrease in the percent of splenic CD3⁺ T cells and CD4⁺ T cells at 14 days p.i. (Table 1). At 21 days p.i. there was also evidence of alternative macrophage activation with a significant increase in the amount of soluble collagen synthesis (Fig 1E) and arginase activity (Fig 1F). Therefore, we selected day 21 p.i. as the time point to establish the ex vivo splenic explant culture so that the screening of small molecules for anti-Leishmania activity would be conducted within the milieu of the immunopathological mechanisms that lead to progressive VL.

Optimization of the ex vivo splenic explant assay for drug screening

For the ex vivo assay to effectively identify compounds that had inhibitory or leishmanicidal activity it was critical that the cultured splenocytes support the replication of the parasite in the absence of active drug over the course of the ex vivo culture. Additionally, since the luciferase-expressing episomal vector could be gradually lost after hamster inoculation, we had to establish the feasibility of quantifying parasite numbers in the ex vivo system. We found that

---

**Table 1. Cellular composition of the splenic explant culture from hamsters infected with L. donovani.**

| Cell type                  | Result 0 | 7 | 14 | 21 |
|----------------------------|---------|---|----|----|
| Splenocytes                | 35.0 ± 10.8 | 86.6 ± 56.6 | 119 ± 23.3 | 135.8 ± 16.0 |
| CD3+ T Cells               | 21.6 ± 5.4 | 23.3 ± 3.7 | 13.6 ± 3.5 | 18.5 ± 0.5 |
| No.                        | 7.6 ± 1.9  | 20.2 ± 3.2  | 16.1 ± 4.2  | 25.1 ± 0.7  |
| CD4+ T Cells               | 5.6 ± 1.9  | 6.9 ± 3.5  | 2.6 ± 0.5   | 6.1 ± 0.9   |
| No.                        | 1.8 ± 0.7  | 6.0 ± 3.0   | 3.1 ± 0.5   | 8.3 ± 1.3   |
| B Lymphocytes              | 14.6 ± 2.0 | 20.3 ± 5.8 | 23.7 ± 4.6  | 18.1 ± 1.3  |
| No.                        | 5.1 ± 0.7  | 17.6 ± 5.0 | 23.8 ± 5.5  | 24.6 ± 1.8  |
| Macrophages                | 15.4 ± 4.3 | 17.4 ± 3.0 | 9.5 ± 2.2   | 23.8 ± 2.6  |
| No.                        | 5.4 ± 1.5  | 12.0 ± 7.1 | 11.4 ± 2.6  | 32.3 ± 3.5  |
| Granulocytes               | 3.15 ± 1.2 | 0.96 ± 0.4 | 1.44 ± 0.2  | 0.46 ± 0.1  |

1 Determined in 5 uninfected hamsters and 5 infected hamsters at each time point.
2 Number of total splenocytes (millions) obtained from the spleen of the animals. Enumerated in a Neubauer chamber by light microscopy.
3 Percentage of positive cells determined by flow cytometry.
4 Number of cells (millions) calculated from the total number of cells multiplied by the percentage of cells positive by flow cytometry.
5 Granulocytes: percentage of granulocytes determined by Side Scatter and Forward Scatter of cells by flow cytometry.

*2*0.001 uninfected vs. infected groups;
*p*<0.01; uninfected vs. infected groups;
*p*<0.05; uninfected vs. infected groups (Kruskal-Wallis test);
*p*<0.001, uninfected vs. infected groups (Tukey-Kramer Multiple comparisons test).

DOI: 10.1371/journal.pntd.0000962.t001

---

The luciferase activity of amastigotes strongly correlated with the number of parasites counted by microscopy at the different times post-infection (R² = 0.99, p < 0.0001; Figure 2A). Luminometry clearly detected amastigote viability in the ex vivo system and most clearly detected amastigote viability in the ex vivo system and most
Figure 2. Characterization of the splenic explant cultures. (A) Representative amastigote standard curve. Correlation between the number of L. donovani amastigotes counted by microscopy and the luciferase activity determined by luminometry. (B) Amastigote replication in splenic explant cultures. Number of amastigotes in ex vivo explants cultures determined by luminometry and interpolation from the standard curve over 0 to 72 hours of incubation (100,000 splenocytes per well). (C) Percent of infected macrophages in splenic ex vivo cultures. Splenocytes harvested from infected hamsters (21 days p.i.) were plated and the proportion of infected macrophages (shown as the mean ± SD) was determined by microscopy at 0, 24, 48, and 72 hours of ex vivo culture. (D) Number amastigotes per 100 macrophages. Amastigotes
between active and inactive compounds. We also found that variation of the ex vivo spleenocyte number from 100,000 to 500,000 cells per well had no effect on the calculated EC_{50} of a test compound (data not shown).

To confirm the quality of the assay in discriminating active from inactive compounds we calculated that the Z prime (Z’ factor [31]) in 3 different screening experiments using 36 different plates. The resulting value of 0.72±0.02 indicated that the assay could be considered as optimal (optimal assay, Z factor ≥0.5 but ≤1.0) [31]. To select the optimal drug concentration for the assays we compared the number of hits obtained after screening 80 different compounds at 10, 5 and 2.5 µM. Each of the concentrations gave similar numbers of hits (data not shown), so we chose the 10 µM concentration to be inclusive of as many hits as possible and limit the skewing of the hit selection toward toxic compounds.

Validation and prioritization of screening hits

The initial screening using the ex vivo model showed that 239 of 4,035 compounds (5.9%) had a Z score of ≤−1.96 (equivalent to a p≤0.05) and could be qualified as screening ‘actives’ (Table 3). We did not observe microscopic evidence of cell death in the splenic explant cultures after 48 hrs of culture with the test compounds. Flow cytometry of propidium iodide stained spleocytes cultured with three representative test compounds (ampotericin B, tilorone, disulfiram) confirmed no loss of spleocyte viability over the 48 hr culture period (data not shown). To quantify cytotoxicity of a test compound we utilized an established cell line-based assay. We first excluded compounds that fell below an arbitrary cytotoxicity threshold in the HepG2 cell line (CC_{50} < 10 µM), and after exclusion of 37 (1%) such (toxic) compounds, 202 (5%) hits were left for further validation studies (Table 3). For each of these hits, anti-leishmanial activity (EC_{50}) was determined in the ex vivo spleenic explant system. Comparison of these EC_{50} values with cytotoxicity (CC_{50}) values in the HepG2 cell line allowed determination of an in vitro therapeutic index (IVTI: CC_{50}/EC_{50}). Based on a threshold IVTI score ≥5 [33], 84 compounds (21% of the total number of molecules screened) were identified as lead compounds (Tables 3, 4, S1). Of the 84 lead compounds, 15 (17%) had been shown previously to have anti-leishmanial activity (Table 4, S1). A substantial number of the latter, however, had been shown previously to have anti-infective activity against other classes of pathogens, while others were known as immune regulators, anti-depressant, antipsychotics, or had no known function (Table 5).

In general, heterocyclic compounds were most highly represented among the lead compounds, comprising 55% of the total (Table 4). Eleven percent of the lead compounds were single-ring heterocyclic structures, 21% had 2-ring structures, 14% were classified as quinolines, and 7% were 3-ring phenothiazines. Ten percent of the leads were alkaloids and 20% were hydrocarbon structures composed of aromatics and terpenes (Tables 4, S1). The chemical libraries screened included large numbers of known bioactives and drugs so it was not surprising that 27 of 84 (32%) leads had been previously used clinically. Eleven of the lead compounds are recommended for topical use only (Table 5).

Three known anti-leishmanial drugs, fluconazole, pentamidine and miltefosine included in the libraries surprisingly did not show a significant Z score and were not identified as hit compounds in the screening. To understand the reason behind this finding, we determined the EC_{50} of these and other anti-leishmanial drugs and lead compounds in both the ex vivo spleen explant and in vitro macrophage infection models (Table 6). Repeated testing of Miltefosine from the NCI chemical library found it to be inactive, but testing of freshly solubilized compound from a different commercial source was found to be highly active (EC_{50} = 1 µM). Thus it would appear that the miltefosine in the NCI library had degraded to an inactive form. Similarly, the EC_{50} calculated for the amphotericin B present in the library (in DMSO vehicle) was 10.7±0.9 µM, whereas freshly solubilized amphotericin B deoxycholate (Sigma) had an EC_{50} of 0.24±0.02 µM. In the case of pentamidine, the Z score of −1.87 was just outside the threshold for statistical significance (a Z score of −1.96 is equivalent to p = 0.05) and determination of the EC_{50} for pentamidine revealed that it was active in the ex vivo spleen explant system (EC_{50} = 3 µM). Fluconazole had no activity detected by either the screen or determination of the EC_{50}. Collectively these data indicate that the ex vivo system is a robust approach to identification of new compounds, but that like any screen, it is only as good as the quality of the compounds (libraries) screened.

Table 2. Determination of spleocyte numbers required for the ex vivo assay.

| No. of cells (per well) | Parasite counts (photons/sec) * | Reduction in parasite burden b |
|-------------------------|-------------------------------|--------------------------------|
|                         | Untreated (control)           | Treated (AMB)                  | (%)                      |
| 3,125                   | 0.7±0.0                       | 0.1±0.1                        | 83.5                     |
| 6,250                   | 1.2±0.2                       | 0.2±0.0                        | 86.3                     |
| 12,500                  | 2.8±0.9                       | 0.4±0.3                        | 86.2                     |
| 25,000                  | 5.3±0.8                       | 0.7±0.0                        | 86.4                     |
| 50,000                  | 14.2±0.4                      | 2.5±0.5                        | 82.3                     |
| 100,000                 | 51.0±10.1                     | 8.5±0.2                        | 83.3                     |
| 200,000                 | 145.2±0.4                     | 34.8±3.7                       | 76.0                     |

*Parasite counts (mean ± SD) were determined by luminometry in spleocytes isolated from hamsters at 21 days p.i. and cultured ex vivo with amphotericin B (AMB), 0.126 µg/ml or DMSO control for 48 h.

bReduction in parasite burden with reference to controls = 100 - [(parasite counts in treated spleocytes/parasite counts in control wells) x 100].

doi:10.1371/journal.pntd.0000962.g002
Comparison of compounds tested in the ex vivo explant and in vitro macrophage infection models

To further validate the ex vivo splenic explant model for drug screening we determined the EC50 of a subset of 10 compounds (including 5 known anti-leishmania drugs) using both the ex vivo and in vitro infected macrophage systems. Infected splenocytes and in vitro infected macrophages were cultured under the same conditions for 48 hrs in the presence or absence of serial dilutions of test compound. We found good correlation between the two systems (R2 = 0.78; p = 0.027).

Discussion

New drugs are desperately needed for the treatment of VL, and innovative approaches are needed to identify new lead compounds and classes of compounds that can enter the pipeline of lead optimization and therapeutic testing. We describe here a novel approach to drug discovery for VL. We sought to develop a model system through which the activity of test compounds could be determined within the physiological and immunological environment of the site of infection. Since the host immune response is known to have profound influence on the treatment and outcome of Leishmania infection, and VL is characterized by suppression of cellular immune function, we felt it was critical that new compounds be screened for activity within the immunopathological milieu found at the site of the host-parasite interaction during active disease. The ex vivo splenic explant culture used for the drug screening reported here was established from L. donovani infected hamsters that demonstrated the immunopathological features of active VL in that 1) there was enlargement of the spleen, 2) the splenic parasite burden was rapidly increasing, 3) there was loss of antigen-specific T cell reactivity [34], 4) the splenic T cell population was contracting while the B cell and macrophage populations were expanding [35], and 5) the splenic macrophages had acquired an alternatively activated phenotype. Furthermore, the cultured spleen cell explants contained the full repertoire of spleen cells and supported ongoing parasite replication during the 48 hrs of exposure to test compound.

The novel ex vivo splenic explant model showed excellent discrimination between active and inactive compounds in a medium-throughput screening format. Known anti-Leishmania drugs were readily identified upon the screening of the chemical libraries, confirming the ability of the ex vivo model to identify active compounds. Furthermore, the practical advantages of the ex vivo approach presented here are numerous. The model uses a Leishmania strain that is episomally transfected with the firefly luciferase reporter gene, which has been successfully used by others to quantify in vitro and in vivo infections [19,36]. The light emission of luciferase-transfected parasites allows one to quantify the amastigote numbers in the samples by extrapolation to a standard amastigote curve, making this approach readily adaptable to a quantitative high throughput assay. The relative high cost of the luciferin substrate is offset by the radical decrease in labor, high sensitivity, and reproducibility of the method. Since in the ex vivo model the cells are harvested from infected animals and the screening is carried out in 96-well plates, no parasitological expertise to quantify amastigotes is necessary, and the time consuming and potentially biased microscopy-based evaluations that preclude automation are avoided. The fact that the luciferase-transfected L. donovani are selected by their resistance to the aminoglycoside G418 (Geneticin) suggests that related compounds would not be identified in the screen, however we found that another aminoglycoside, neomycin, was identified as a compound with activity against the transgenic L. donovani used in the ex vivo system. Other approaches that have used GFP-transfected Leishmania showed that the sensitivity of measuring fluorescence is not sufficient to enable microplate screening, and consequently a more demanding analysis using a flow cytometer is required [37]. The requirement of animals to establish the ex vivo splenic explant model is minimal because the cells obtained from a single infected hamster are sufficient to screen more than 1,000 compounds.

Although axenically cultured amastigotes have been used to screen drug candidates on a small scale [38,39] not all parasite strains can be cultured as axenic amastigotes. It has been shown that in vitro assays that utilize intracellular amastigotes in macrophage cell lines correlate better with the response to treatment in vivo compared with assays in which promastigotes are used [40]. Screenings that involve in vitro infected macrophages have the technical limitation of difficulty in removal of extracellular promastigotes, and the theoretical concern that the non-macrophage immune regulatory cells are absent. Our results showed that the anti-Leishmania activity of lead compounds in the ex vivo spleen cell explants differed substantially from that found in cultured promastigotes, and to a lesser extent from freshly isolated tissue amastigotes. Thus, some of the lead compounds would not have been identified if promastigotes or cell-free

Table 3. Hit and lead compounds identified from chemical libraries using the ex vivo splenic explant system.

| Compounds | Initial Screening | Results after excluding toxic compounds* |
|-----------|------------------|----------------------------------------|
| Chemical Library | Z score <−1.96 6 | Hits 4 No. | Leads (IVTI>5) 7 |
| NINDS Col II1 | 1,040 | 76 | 65 | 24 |
| NINDS NP 2 | 800 | 51 | 46 | 12 |
| NCI 3 | 2,195 | 112 | 91 | 48 |
| Total 4 | 4,035 | 239 | 202 | 84 |

1NINDS Col II (NINDS Collection II, MicroSource Discovery Systems).
2NINDS NP (NINDS Natural Products Collection, MicroSource Discovery Systems).
3Diversity set and Natural products set of the National Cancer Institute (NCI).
4Compounds identified by Z score, excluding the toxic compounds (CC50 <10 μM for the HepG2 cell line).
5Total number (No.) and % of compounds excluding amphotericin B, which is considered as the reference compound.
6All percentages shown in the table refer to the total number of compounds of the initial screening. See Table S1 for compound details.
7Leads identified using the in vitro therapeutic index (IVTI) calculated of the cell toxicity (CC50) and anti-Leishmania activity (EC50) ratio.

*Comparison of compounds tested in the ex vivo explant and in vitro macrophage infection models.

doi:10.1371/journal.pntd.0000962.t003
Table 4. Lead compounds newly identified or previously known to have anti-leishmanial activity.

| Compound 1 | New | Known | Total |
|------------|-----|-------|-------|
| Heterocyclic compounds |     |       |       |
| 1-ring | | | |
| Furan | 3 | 4 | 1 | 7 | 4 | 5 |
| Pyridines | 1 | 1 | 0 | 0 | 1 | 1 |
| Piperidines | 1 | 1 | 0 | 0 | 1 | 1 |
| Pyran | 0 | 0 | 3 | 20 | 3 | 4 |
| 2-ring | | | |
| Isoquinolines | 2 | 3 | 0 | 0 | 2 | 2 |
| Quinolines | 12 | 17 | 0 | 0 | 12 | 14 |
| Purines | 1 | 1 | 0 | 0 | 1 | 1 |
| Benzopyrans | 2 | 3 | 0 | 0 | 2 | 2 |
| Bicyclocomp. | 1 | 1 | 0 | 0 | 1 | 1 |
| 3-ring | | | |
| Phenothiazines | 5 | 7 | 1 | 7 | 6 | 7 |
| Acridines | 1 | 1 | 0 | 0 | 1 | 1 |
| Xanthenes | 3 | 4 | 0 | 0 | 3 | 4 |
| Phenanthridines | 0 | 0 | 1 | 7 | 1 | 1 |
| Alkaloids | 9 | 13 | 2 | 13 | 11 | 13 |
| Hydrocarbons | | | |
| Aromatics | 8 | 12 | 1 | 7 | 9 | 11 |
| Terpenes | 6 | 9 | 1 | 7 | 7 | 8 |
| Acyclic | 1 | 1 | 0 | 0 | 1 | 1 |
| Polycyclic compounds | | | |
| Macrocyclic | 1 | 1 | 1 | 7 | 2 | 2 |
| Steroids | 1 | 1 | 2 | 13 | 3 | 4 |
| Amines | 2 | 3 | 3 | 20 | 5 | 6 |
| Ethylamines | 1 | 1 | 0 | 0 | 1 | 1 |
| Q. ammonium | 2 | 9 | 0 | 0 | 2 | 2 |
| Polyamines | 1 | 1 | 0 | 0 | 1 | 1 |
| Lactones | 4 | 6 | 0 | 0 | 4 | 5 |
| Onium comp. | 1 | 1 | 2 | 13 | 3 | 4 |
| Sulfur comp. | 2 | 3 | 3 | 1 | 7 | 3 | 4 |
| Other | 3 | 4 | 1 | 7 | 4 | 5 |
| Total | 69 | 82 | 15 | 18 | 84 | 100 |

1Chemical name according the MeSH Chemical Class Browser (Wolfram Demonstrations project), National Library of Medicine, National Institutes of Health, United States 2010.
2Others: Amidines, Phenols, Carboxilic Acids, Organometallic compounds.

doi:10.1371/journal.pntd.0000962.t004

amastigotes had been used. The lack of correlation between these techniques suggests that this ex vivo screening system can be exploited for the discovery of drugs targeting metabolic pathways of amastigotes in the host cells and/or interacting with the immune system. Accordingly, review of the published literature about the mechanisms of action of the 84 lead compounds revealed that the likely mode of action or parasite targets were the cell membrane (21%), cell metabolism (17%), the host immune response (13%), apoptosis (7%), and DNA interaction (6%).

The screening of compounds for anti-Leishmania activity in the ex vivo model, coupled with screening for toxicity using the HepG2 hepatocyte cell line, enabled the selection of 84 lead compounds, 69 of which had not been identified previously to have anti-Leishmania activity. While the use of cell lines to predict in vivo toxicity has some limitations [41], the HepG2 cell line is considered a good predictor of human toxicity [42,43]. Although measurement of ATP in viable cells this method is one of the most reliable methods to estimate cell toxicity [44] multiparametric toxicity testing (based on features such as apoptosis markers and membrane integrity) and other in vitro models may be desirable to help in the assessment of hepatic cytotoxicity [45] and further lead optimization [46]. Ultimately, the final selection of lead compounds will require in vivo studies to identify dose limiting toxicities and to evaluate whether the compound’s pharmacokinetic and ADME (absorption/distribution/metabolism/excretion) properties make it suitable for use in VL. Already it is clear that some of the lead compounds (e.g. topical and antiseptic agents) are unlikely to be useful in treating systemic infection.

Compounds containing the heterocyclic quinoline ring system were frequently identified in the screen as active inhibitors, representing 14% of the lead compounds. The quinoline leads could be further divided into several distinct classes. A number of the leads bear obvious structural relation to antimalarial quinolines, including several 4-aminoquinolines with basic side chains, as well as 8-aminoquinolines and a 2-arylquinoline derivative containing the core structure of the antimalarial drug mefloquine. Also represented were 8-hydroxyquinoline antifungals like clioquinol as well as more novel dimeric 2-aminoquinolines and quinoline diones. Several of the quinoline-containing compounds showed activity at micromolar concentrations and low toxicity to the HepG2 cell line, suggesting that they are good leads for optimization. Previous reports have described other quinoline-containing compounds with anti-Leishmania activity. For example, a 2-substituted quinoline alkaloid reduced by 79.6% the parasite load in the liver of BALB/c mice infected with L. donovani [46]. Sittamaquine, an 8-aminoquinoline, completed a phase II study for treatment of VL [47], and more recently, DNDI has incorporated synthetic 2-quinoline derivatives as part of the strategy to develop new anti-Leishmania drugs (http://www.dndi.org/newsletters/n18/edito.php). The quinoline ring system is common in known drugs and other bioactive molecules and such compounds can exhibit distinct bioactivities depending on their specific structures. Previously, quinoline derivatives have been reported to affect electron transport and generate lethal oxidative radicals against Leishmania, and also to inhibit cysteine proteases [48], a gene family important for Leishmania virulence [49]. Other quinoline derivatives have also been shown to alter the vesicle trafficking and endocytosis in Plamodium falciparum [50]. Our results, together with these prior observations, suggest the likely possibility that multiple distinct bioactivities are represented among the quinoline leads identified here and that multiple pharmacologically orthogonal candidates might be selected for further lead optimization studies.

Alkaloids also represented an important fraction of the lead compounds (10%) identified in the ex vivo screening. This parallels the observation that alkaloids derived from natural products have been found to be active against Leishmania species (reviewed by [51]).
Table 5. Therapeutic category of anti-leishmanial compounds identified in the ex vivo splenic explant system.

| Anti-protozoa | Anti-bacterial | Anti-helminthic |
|---------------|---------------|----------------|
| Ellipticine    | Nonactin      | Parasanoamine Pamoate |
| Nigercin      | Aklavine Hydrochloride | Naphthofuran  4 |
| Cepharanthine | Nigercin      | Cetrominium Bromide |
| Disulfiram    | Naphthofuran  4 | Disulfiram |
| Tilorone      | Streptovitacin A | 3-phenanthren 6 |
| Benzalkonium Chloride | Securinine | Parasanoamine Pamoate |
| Physalin      | Cepharanthine | Naphthofuran 4 |
| Chlorhexidine | Parthenin      | Cetrominium Bromide |
| Cloxyquin     | Disulfiram    | Disulfiram |
| Tetrandrine   | Salinomycin, Sodium | Acrisicin |
| Cloqunol      | Rubescensin A | Benzalkonium Chloride |
| Hexachlorophore | 7-hydroxyloromazine | Clioquinol |
| Lasalocid Sodium | Lasalocid Sodium | Hexachlorophene |
| Anti-neoplastic | Topical/Antiseptics | Antimycin A |
| Aklavine Hydrochloride | 5-methyl-8-quinolinol 6 | 5-methyl-8-quinolinol 6 |
| Thaspine, Acetate | Cetrominium bromide | Immune response regulator |
| Ellipticine    | Benzalkonium chloride | Immune response regulator |
| NSC 134754    | Chlorhexidine | n6-isopentenyladenine |
| Streptovitacin A | Clioquinol | Spermidin Trihydrochloride |
| Cepharanthine | Hexachlorophore | NSC 13480 |
| Benzethonium Chloride | Cetylpyridinium Chloride | 6,4’-Dimethoxyflavone |
| Rubescensin A | NSC 371488 | |
| 10-Methyl-9-anthracenyl | Anti-viral | Sumilibt bbm |
| Tilorone      | Nigercin      | Securinine |
| Crassin      | Cepharanthine | Cepharanthine |
| Tetrandrine   | Trimethoxychalcone 9 | |
| NSC 305189    | Tilorone      | Anti-obesity/Anti-psychotics |
| NSC 371488    | Benzalkonium Chloride | 1h-benzedeisouquinoline  b |
| Cloquinol     | Crassin Acetate | Nortriptyline Hydrochloride |
| Fastigilin B  | Disulfiram    | |
| Thioxanthen-9  | Undefined  b | Orlistat |
| Crassin acetate | Thiomethylpromazine | |
| 6,4’- dimethoxyflavone | Chlorpromazine | |
| 7-hydroxychlorpromazine | Maprotionate hydrochloride | |

*3-phenanthrenemethanol, alpha-3(diethylamino)methyl)-hydrochloride.  
1h-benzedeisouquinoline-1,3(2-h)-dione, 5-amine-2-2[2-diethylaminoethyl]-7-2-(6-ethoxy-1-methyl-1-tialbalka(5)-quinolin-2-yllvinyl)-5-methyl-8-quinolinol.  
Naphthofuran-4, 4 methoxy-2-nitro.  
Thioxanthen-9-one, 4-hydroxyxymethyl-1-[2-piperidinoethyl]amino.  
10-Methyl-9-anthracenyl methyl carbamimidothioic acid ester hydrochloride.  
2,4-dihydroxy-3,4’-2 Trimethoxychalcone.  

Disruption or alteration of membrane function was identified as a mode of action for a number of compounds (ionophores, quaternary ammonium salts and tricyclic anti-depressants [52]) that were identified as leads in our screen (Tables 4, S1). In addition to a possible direct effect on the parasite membrane these inhibitors have the potential to affect the membrane integrity of the parasitophorous vacuole in which Leishmania resides, and perturb the capability to regulate the intraphagosomal trafficking of essential substrates for parasite survival [53]. Specific inhibition of biosynthesis of phosphatidylcholine has been proposed for some quaternary ammonium salts active against L. major promastigotes and L. braziliensis [54]. One of the leads identified here, cetrominium bromide, is a cationic surfactant that closely resembles the structure of hexadecylphosphocholine (miltefosine), a drug in current use for VL. It has also been shown to inhibit choline kinase that regulates the biosynthesis of the most abundant phospholipid (phosphatidylcholine) in Plasmodium falciparum [55]. Because these lead compounds are recommended only for topical and not systemic administration they will not be drug candidates for VL, but identification of their molecular targets could facilitate new screening campaigns that could identify lead compounds more suitable for systemic use.

Phenothiazine compounds related to the tricyclic antidepressants constituted 7% of the leads, but their favorable IVTI was primarily a result of their low toxicity, rather than their good anti-leishmanial activity (EC50, 11 ± 2.7 μM). This class of drugs was identified as having in vitro anti-Leishmanial activity 30 years ago, but the absence in the literature of any in vivo therapeutic data would suggest that may not have good clinical efficacy. Other phenothiazine compounds are potent inhibitors of parasite trypanothione reductase, a key enzyme involved in many redox defenses of Leishmania. However, no correlation between anti-leishmanial efficacy and the potency of several trypanothione reductase inhibitors was found [56].

A broad range of biological effects have been recognized for the hydrocarbon terpenes, which represented 8% of the lead compounds. After alkaloids, natural product terpenes are the inhibitors found most frequently as having activity against Leishmania spp. [51]. The terpenoids of the plant family Asteraceae pathenolide have been shown to inhibit amastigotes and promastigotes of L. amazonensis [57]. An analog of Harmine, a betacaroline amine alkaloid identified in our screening, reduced the spleen parasite load by 40–80% in hamsters through necrosis and non-specific parasite membrane damage [58]. However, the selection of inhibitors targeting parasite specific metabolic pathways without altering the host’s cell would be more desirable.
Table 6. Comparative anti-*Leishmania donovani* activity of lead compounds in the ex vivo splenic explant model and in vitro infected peritoneal macrophages.

| Compound                | Ex vivo model EC₅₀ (μM; mean±SE)¹ | Peritoneal Macrophages EC₅₀ (μM; mean±SE)¹ |
|-------------------------|-----------------------------------|-----------------------------------------------|
| Amphotericin B          | 0.09±0.03                         | 0.11±0.01                                       |
| Miltefosine             | 2.02±0.58                         | 2.28±0.01                                       |
| Pentamidine             | 3.02±0.34                         | 0.09±0.37                                       |
| Fluconazole             | >250                              | >250                                            |
| Meglumine antimoniate   | 269.60±57.50                      | 146.30±42.01                                    |
| Antimycin A             | 1.27±1.07                         | 1.67±1.05                                       |
| Disulfiram              | 0.31±0.03                         | 0.16±0.03                                       |
| Monensin A              | 0.85±0.68                         | 0.23±0.15                                       |
| Nortriptyline           | 6.03±0.28                         | 2.65±0.35                                       |
| Tilorone                | 0.84±0.47                         | 1.48±0.35                                       |

¹Effective concentration of the compound that killed 50% of the parasites (EC₅₀). Determined at 48 h of culture by luminometry. doi:10.1371/journal.pntd.0000962.t006

In summary, the ex vivo splenic explant model, which is comprised of the full repertoire of host cells including chronically infected macrophages and fibroblasts, enabled the identification of small molecules that have anti-*Leishmania* activity within the immunopathological milieu that closely resembles the in vivo features of progressive VL. The inclusion of the complex biological interactions between the parasite and host within the test system may also favor the identification of lead compounds that act at multiple targets [59]. The standardized approach presented here identified a number of compounds that had good potency, including some that are currently in clinical use for other indications. Further study in animal infection models seems a prudent next step for those agents already used systemically. Among the other lead compounds identified in this work are several interesting chemotypes (e.g. quinolines) that are good candidates for a lead optimization. The identification of highly active anti-leishmanial compounds in this ex vivo model of VL could contribute greatly to new drug discovery for this serious and neglected disease.

References

1. Croft SL, Sundar S, Fairlamb AH (2006) Drug resistance in leishmaniasis. Clin Microbiol Rev 19: 111–126.
2. Haendlein E, Keszler I, Udolf AD, Goding JW (2008) Fishing for anti-leishmania drugs: principles and problems. Adv Exp Med Biol 625: 48–60.
3. Ephros M, Binum A, Shaked P, Waldman E, Zilberstein D (1999) Stage-specific activity of pentavalent antimony against Leishmania donovani axenic amastigotes. Antimicrob Agents Chemother 43: 279–282.
4. Valithan R, Dubez M, Mahajan RC, Malla N (2006) Leishmania donovani: effect of verapamil on in vitro susceptibility of promastigote and amastigote stages of Indian clinical isolates to sodium stibogluconate. Exp Parasitol 114: 103–108.
5. Murray HW, Granger AM, Mohanty SK (1991) Response to chemotherapy in experimental visceral leishmaniasis: T cell-dependent but interferon-gamma-and interleukin-2-independent. J Infect Dis 163: 622–624.
6. Murray HW, Montelhano C, Peterson R, Sypek JP (2000) Interleukin-12 regulates the response to chemotherapy in experimental visceral Leishmaniasis. J Infect Dis 182: 1497–1502.
7. Murray HW, Oca MJ, Granger AM, Schreiber RD (1989) Requirement for T cells and effect of lymphokines in successful chemotherapy for an intracellular infection. Experimental visceral leishmaniasis. J Clin Invest 83: 1253–1257.
8. Alexander J, Carter KC, Al-Fasi N, Satoskar A, Bormbach L (2000) Endogenous IL-4 is necessary for effective drug therapy against visceral leishmaniasis. Eur J Immunol 30: 2933–2943.
9. Sacks D, Anderson C (2004) Re-examination of the immunosuppressive mechanisms mediating non-cure of Leishmania infection in mice. Immunol Rev 201: 223–238.
10. Gifweswes C, Farrell JP (1989) Comparison of T-cell responses in self-limiting versus progressive visceral Leishmania donovani infections in golden hamsters. Infect Immun 57: 3091–3096.
11. Melby PC, Chandrasekar B, Zhao W, Coe JE (2001) The hamster as a model of human visceral leishmaniasis: progressive disease and impaired generation of nitric oxide in the face of a prominent Th1-like response. J Immunol 166: 1912–1920.
12. Melby PC, Tryon VV, Chandrasekar B, Freeman GL (1998) Cloning of Syrian hamster (Mesocricetus auratus) cytokine cDNAs and analysis of cytokine mRNA expression in experimental visceral leishmaniasis. Infect Immun 66: 2135–2142.
13. Kenney RT, Sacks DL, Gam AA, Murray HW, Sundar S (1998) Splenic cytokine responses in Indian kala-azar before and after treatment. J Infect Dis 177: 815–818.
14. Kaup CL, El-Saif SH, Wynn TA, Satti MM, Kordofani AM, et al. (1993) In vivo cytokine profiles in patients with kala-azar. Marked elevation of both interleukin-10 and interferon-gamma [see comments]. J Clin Invest 91: 1644–1648.
15. Murray HW, Teitelbaum RF (1992) L-arginine-dependent reactive nitrogen intermediates and the antimicrobial effect of activated human mononuclear phagocytes. J Infect Dis 165: 513–517.
16. Denis M (1994) Human monocytes/macrophages: NO or NO? J Leuk Biol 55: 682–684.
17. Perez EE, Chandrasekar B, Saldañia OA, Zhao W, Arteaga LT, et al. (2006) Reduced nitric oxide synthase 2 (NOS2) promoter activity in the Syrian hamster renders the animal functionally deficient in NOS2 activity and unable to control an intracellular pathogen. J Immunol 176: 5519–5528.

Supporting Information

Table S1. Lead compounds with anti-*Leishmania donovani* activity identified in the screening using the ex vivo splenic explant model.

Table S2. Lead compounds known to have anti-*Leishmania* activity identified by screening in the ex vivo splenic explant model.

Author Contributions

Conceived and designed the experiments: YO BLT PCM. Performed the experiments: YO AGP. Analyzed the data: YO BLT ARR AGP PCM. Wrote the paper: YO BLT ARR AGP PCM.
10. Sacks D, Melby P. [1998] Animal models for the analysis of immune responses to leishmaniasis. In: Coligan J, Kruisbeek A, Margulies D, Shevach E, Strober W, eds. Current Protocols in Immunology: John Wiley and Sons, Inc. 19.12.11–19.12.20.
11. Roy G, Dumas C, Sereno D, Wu Y, Singh AK, et al. (2000) Epithelial and submucosal expression of the luciferase reporter gene for quantifying Leishmania spp. infections in macrophages and in animal models. Mol Biochem Parasitol 110: 195–206.
12. Corraliza IM, Campo ML, Soler G, Modolell M (1994) Determination of arginase activity in macrophages: a micromethod. J Immunol Methods 174: 231–235.
13. Melby PC, Yang YZ, Cheng J, Zhao W (1998) Regional differences in the cellular immune response to experimental cutaneous or visceral infection with Leishmania donovani. Infect Immun 66: 18–27.
14. Lim LC, Englund DM, Glowacki NJ, DuChateau BK, Schell RF (1995) Involvement of CD4+ T lymphocytes in induction of severe destructive Lymnae arthritis in inflamed LSH hamsters. Infect Immun 63: 4818–4825.
15. Liu H, Seinan BM, Aldor JD, Baertschy DK, Schell RF (1990) Immune T cells sorted by flow cytometry confer protection against infection with Treponema pallidum subsp. pertenue in hamsters. Infect Immun 58: 1085–1090.
16. Heemskerk J, Tobin AJ, Ravina B (2002) From chemical to drug: neurodegeneration drug screening and the ethics of clinical trials. Nat Neurosci 5 Suppl: 1027–1029.
17. Piacentini F, Roman BR, Fischer KH, Taylor JP (2004) A screen for drugs that protect against the cytotoxicity of polyglutamate-expanded androgen receptor. Hum Mol Genet 13: 437–446.
18. Rothstein JD, Patel S, Regan MR, Haeggeli C, Huang YH, et al. (2005) Beta-lactam antibiotics offer neuroprotection by increasing glutamate transporter expression. Nature 433: 73–77.
19. Glover CJ, Hite KD, DeLosh R, Scudiero DA, Fivash MJ, et al. (2003) A high-throughput screen for identification of molecular mimics of Smac/DIABLO utilizing a fluorescence polarization assay. Anal Biochem 320: 157–169.
20. Marx C, Berger C, Xu F, Amend C, Scott GK, et al. (2006) Validated high-throughput screening of drug-like small molecules for inhibitors of EthR2 transcription. Assay Drug Dev Technol 4: 273–284.
21. Rapisarda A, Uranchimeg B, Scudiero DA, Melby S, Sausville EA, et al. (2002) Identification of small molecule inhibitors of hypoxia-inducible factor 1 transcriptional activation pathway. Cancer Res 62: 4316–4324.
22. Malo N, Hanley JA, Cerquozzi S, Pelletier J, Nadon R (2006) Statistical practice in high-throughput screening data analysis. Nat Biotechnol 24: 167–175.
23. Zhang JH, Chung TD, Oldenburg KR (1999) A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. J Biomol Screen 4: 67–73.
24. Rass TL, Moravec RA (2004) Use of multiple assay endpoints to investigate the effects of incubation time, dose of toxin, and plating density in cell-based cytotoxicity assays. Assay Drug Dev Technol 2: 51–62.
25. Duan H, Takaishi Y, Imakura Y, Jia Y, Li D, et al. (2000) Sesquiterpene lactone isolated from Tanacetum parthenium. Antimicrob Agents Chemother 44: 2588–2594.
26. Illi G, Harmine: evaluation of its antileishmanial properties in various vesicular transport assays. Parasitol Int 54: 103–109.
27. Saravia NG, Escorcia B, Olorio Y, Valderrama L, Brooks D, et al. (2006) Role of monoquaternary ammonium derivatives in the treatment of visceral leishmaniasis caused by Leishmania chagasi. Am J Trop Med Hyg 65: 685–689.
28. Koyama J (2006) Anti-infective quinone derivate of recent patents. Recent Pat Antiinfect Drug Discov 1: 113–125.
29. Roberts L, Egan TJ, Joiner KA, Hoppe HC (2008) Differential effects of quinoline antiinfectives on endothelial cell injury. Antiinfect Drug Discov 1: 1040–1043.
30. Rocha LG, Almeida JR, Macedo RO, Barbosa-Filho, JM (2005) A review of natural products with antileishmanial activity. Phytochemistry 62: 314–315.
31. Zilberstein D, Dwyer DM (1984) Antidepressants cause lethal disruption of the mammalian peripheral catecholaminergic system. Int 56: 3–7.
32. Dambach DM, Andrew BA, Moulin F (2005) New technologies and screening strategies for hepatotoxicity: use of in vitro models. Toxicol Pathol 33: 17–26.
33. Lala S, Pramanick S, Mukhopadhyay S, Banerjee S, Basu MK (2004) Harmine: evaluation of its antileishmanial properties in various vesicular delivery systems. J Drug Target 12: 163–175.
34. Lira R, Sundar S, Mahakaria A, Kenney R, Gam A, et al. (1999) Evidence that the high incidence of treatment failures in Indian kala-azar is due to the emergence of antimony-resistant strains of Leishmania donovani. J Infect Dis 180: 564–567.
35. Saravia NG, Escorcia B, Olorio Y, Valderrama L, Brooks D, et al. (1994) Pathogenicity and protective immunogenicity of cytochrome proteinase-deficient mutants of Leishmania mexicana in non-murine models. Vaccine 24: 4247–4259.
36. Heemskerk J, Tobin AJ, Ravina B (2002) From chemical to drug: neurodegeneration drug screening and the ethics of clinical trials. Nat Neurosci 5 Suppl: 1027–1029.
37. Piccion F, Roman BR, Fischer KH, Taylor JP (2004) A screen for drugs that protect against the cytotoxicity of polyglutamate-expanded androgen receptor. Hum Mol Genet 13: 437–446.
38. Rapisarda A, Uranchimeg B, Scudiero DA, Melby S, Sausville EA, et al. (2002) Identification of small molecule inhibitors of hypoxia-inducible factor 1 transcriptional activation pathway. Cancer Res 62: 4316–4324.
39. Malo N, Hanley JA, Cerquozzi S, Pelletier J, Nadon R (2006) Statistical practice in high-throughput screening data analysis. Nat Biotechnol 24: 167–175.
40. Zhang JH, Chung TD, Oldenburg KR (1999) A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. J Biomol Screen 4: 67–73.
41. Rass TL, Moravec RA (2004) Use of multiple assay endpoints to investigate the effects of incubation time, dose of toxin, and plating density in cell-based cytotoxicity assays. Assay Drug Dev Technol 2: 51–62.
42. Duan H, Takaishi Y, Imakura Y, Jia Y, Li D, et al. (2000) Sesquiterpene alkaloids from Tripterygium hypoglaucum and Tripterygium willdorii: a new class of potent anti-HIV agents. J Nat Prod 63: 357–361.
43. Dils DK, Lal SL, Shrivastava SN, Blackwell J, Neva FA (1987) Expression of the luciferase reporter gene for quantifying Leishmania spp. infections in macrophages and in animal models. Mol Biochem Parasitol 110: 195–206.