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Identifying inhibitors of the *Leishmania* inositol phosphorylceramide synthase with antiprotozoal activity using a yeast-based assay and ultra-high throughput screening platform

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Leishmaniasis is a Neglected Tropical Disease caused by the insect-vector borne protozoan parasite, *Leishmania* species. Infection affects millions of the world’s poorest, however vaccines are absent and drug therapy limited. Recently, public-private partnerships have developed to identify new modes of controlling leishmaniasis. Drug discovery is a significant part of these efforts and here we describe the development and utilization of a novel assay to identify antiprotozoal inhibitors of the *Leishmania* enzyme, inositol phosphorylceramide (IPC) synthase. IPC synthase is a membrane-bound protein with multiple transmembrane domains, meaning that a conventional *in vitro* assay using purified protein in solution is highly challenging. Therefore, we utilized *Saccharomyces cerevisiae* as a vehicle to facilitate ultra-high throughput screening of 1.8 million compounds. Antileishmanial benzazepanes were identified and shown to inhibit the enzyme at nanomolar concentrations. Further chemistry produced a benzazepane that demonstrated potent and specific inhibition of IPC synthase in the *Leishmania* cell.

The Neglected Tropical Disease (NTD) leishmaniasis is endemic in over 90 countries worldwide, affecting approximately 12 million people per year with 350 million people living at risk of disease. The causative agent, *Leishmania* species, are sandfly borne kinetoplastid protozoan parasites. Infection affects millions of the world’s poorest, however vaccines are absent and drug therapy limited. Recently, public-private partnerships have developed to identify new modes of controlling leishmaniasis. Drug discovery is a significant part of these efforts and here we describe the development and utilization of a novel assay to identify antiprotozoal inhibitors of the *Leishmania* enzyme, inositol phosphorylceramide (IPC) synthase. IPC synthase is a membrane-bound protein with multiple transmembrane domains, meaning that a conventional *in vitro* assay using purified protein in solution is highly challenging. Therefore, we utilized *Saccharomyces cerevisiae* as a vehicle to facilitate ultra-high throughput screening of 1.8 million compounds. Antileishmanial benzazepanes were identified and shown to inhibit the enzyme at nanomolar concentrations. Further chemistry produced a benzazepane that demonstrated potent and specific inhibition of IPC synthase in the *Leishmania* cell.

A vaccine to prevent leishmaniasis is not currently available and treatment relies entirely on a limited number of chemotherapeutics with, in the most part, unclear modes of action. For example, cutaneous leishmaniasis (CL, e.g. caused by *L. major*) therapy largely relies on two pentavalent antimonials, sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime). Despite their problems, including severe side-effects such as cardiotoxicity and the requirement for parenteral administration, Pentostam and Glucantime have been in clinical use for more than 70 years. The treatment options for visceral leishmaniasis (VL, Kala-Azar; largely caused by *L. donovani*) suffer from similar problems, with antimonial therapy still widely employed. In addition, resistance to these drugs is rising and threatening their continued use in the treatment of leishmaniasis. Amphotericin B (Fungizone) has also been used in the treatment of both CL and VL. However, this is also associated with severe side-effects, and must be administered parenterally. Miltefosine, is the only orally available antileishmanial and has been used to treat VL in India since 2002, however, this antineoplastic compound also exhibits teratogenicity...
and resistance is increasingly apparent. Therefore, the current elimination programme in South Asia relies upon liposomal amphotericin B administered as a single injection. However, whilst effective and less toxic than the non-liposomal formulation, treatment failure and post-Kala-Azar dermal leishmaniasis (PKDL) may preclude elimination.

With these severe problems in the treatment and control of both CL and VL, it is recognized that new antileishmanial targets and drugs need to be identified. High throughput screening (HTS) is a vital component of drug discovery and can be carried out using either in vitro assays against validated targets or in phenotypic assays against the parasite itself. Recent high content phenotypic screening across the pathogenic kinetoplastids gave a disappointingly low number of novel potent hits against Leishmania donovani when compared with the related parasites Trypanosoma brucei and T. cruzi. This demonstrated the importance of target-based screening for antileishmanial discovery to access to novel chemical space and new modes of action which may overcome resistance and be compatible in combination therapies.

In contrast to mammalian cells, where the predominant complex sphingolipid is sphingomyelin (SM), fungi, plants and some protozoa synthesize inositol phosphorylceramide (IPC)\(^{15,16}\). The fungal IPC synthase (AUR1p) has long been established as a drug target for pathogenic fungi\(^{17}\). Similarly, the Leishmania orthologue, and those from the pathogenic Trypanosoma species, have been suggested to be ideal, non-mammalian, targets for the development of new, less toxic, antiprotozoals\(^{13,19,20}\). Furthermore, systems biology studies have reinforced the status of the Leishmania enzyme as a putative target for drug discovery programmes\(^{21}\). However, as an integral membrane protein with 6 transmembrane domains, and lipid substrates (phosphatidylinositol and ceramide) and products (diacylglycerol and IPC), formatting the Leishmania IPC synthase into a conventional in vitro assay platform is challenging\(^{22}\). Therefore, utilizing the ability of the kinetoplastid enzyme to complement for the absence of the Saccharomyces cerevisiae orthologue AUR1p\(^{23}\), here we describe the development and formatting of a robust yeast-based ultra-HTS (uHTS) assay platform. This was then utilized, in the largest effort of its type, to screen a high content (1.8 M) compound library for specific Leishmania IPC synthase inhibitors. 500 potent and specific such compounds were identified; these were then reduced to 211 following clustering to remove structural replicates. Following screening against mammalian-stage axenic amastigote L. donovani, 25 of these compounds were selected on the basis of potency, selectivity and physicochemical properties. These hits were then reduced to five following further screening of L. donovani infected macrophages. From these, one pair of structurally related compounds, the benzazepanes, was selected for further analyses. Importantly, these compounds demonstrated sub-micro-molar activity against the enzyme target in a secondary in vitro assay and selectivity for the enzyme in cellulo when using an available L. major sphingolipid mutant\(^{24}\). This work demonstrated the tractability of yeast-vehicles for uHTS, with the identification of the benzazepanes as potential antileishmanials with specific inhibitory activity against IPC synthase.

Results

Design and validation of a robust Leishmania IPC synthase assay for uHTS. HTS campaigns predominantly rely on two approaches: (i) in vitro target-directed screening using biochemical assays; (ii) cell-based phenotypic screening which takes no account of the target. Both of these approaches have significant limitations, such as the production of soluble protein and a lack of cellular context for biochemical assays, and the problems of process redundancy in cells used for phenotypic screening. Furthermore, both can be difficult to miniaturize and expensive to utilize for uHTS. With these problems in mind, yeast-based systems have been utilized to provide cost-effective, target-directed, screening platforms for protein targets within a eukaryotic cellular context. Recently, this approach has been adopted for antiprotozoal drug discovery\(^{13,25,26}\).

The transmembrane nature of the target Leishmania IPC synthase, and the hydrophobicity of the lipid substrates and products, rendered it challenging to develop a uHTS biochemical assay\(^{18,19,22}\). However, given that the enzyme has been shown to complement an auxotrophic Saccharomyces cerevisiae mutant\(^{18}\), the development of yeast-based screening platform was considered tractable. To develop a robust assay suitable for uHTS, rather than the previously utilized auxotrophic mutant\(^{18}\), a strain of S. cerevisiae completely lacking the yeast IPC synthase, AUR1p, was selected as the vehicle (a kind gift from Teresa Dunn, Uniformed Services University)\(^{27}\). This strain is reliant on the expression of essential AUR1p from a uracil selectable expression plasmid (pRS316-URA-AUR1)\(^{27}\). Transforming these with the plasmid pESC-LEU (Agilent) harbouring the L. major IPC synthase (LmjFIPCS) or AUR1p under the control of a galactose promoter, allowed for curing the yeast of pRS316-URA-AUR1 using the pyrimidine analogue 5-fluoro-orotic acid (5-FOA) and the selection of a strain dependant on the galactose inducible expression of LmjFIPCS (Fig. 1). The yeast dependant on either LmjFIPCS or AUR1p expression were then used to format an LmjFIPCS assay suitable for uHTS.

Exploiting the fact that growing yeast secretes exo-β-1,3-glucanase, which, as well as modifying the cell wall, can also hydrolyse non-fluorescent fluorescein di-(β-D-glucopyranoside) (FDGlu) to release fluorescein, an assay was developed in which cell growth was monitored by fluorescein output\(^{28}\). The optimal concentrations of galactose (0.1%), starter culture (OD\(_{600}\) 0.063) and FDGlu (10 μM) were established in 96- and 384-well formats incubated at 30 °C for 24 hours. The assay was then miniaturized into a 1536-well format, the most appropriate positive control (cycloheximide) selected and the robustness of the platform indicated by Z’ values >0.5\(^{29}\). Screening using a GSK standard set of (9766) compounds further validated the assay, established inhibition threshold values and indicated a hit-rate of 1.07%.

Identification of selective and potent inhibitors. Taking the robust 1536-well format assay developed, a primary uHTS screening was conducted using the 1.8 million compound GSK library against the LmjFIPCS dependent yeast. Given the hit-rate of 1.07% with the standard set approximately 19000 hits were anticipated. Initially this expectation was not reached, however reducing the inhibition threshold to capture small (≤300 Da
molecular weight), polar (cLogP ≤ 3) ‘drug-like’ compounds with lower efficacy, led to the identification of 19,669 hits. The mean Z’ for the screen was 0.70, demonstrating the platform to be robust.

At this stage, it was unclear whether the hit compounds inhibited the LmjF IPS enzyme or (an)other essential process(es) within the yeast vehicle. Therefore, a counter screen was performed against the yeast dependant on AUR1p expression at a single compound concentration of 10 μM. In parallel, a confirmatory screen against the LmjF IPS dependent yeast was performed at the same concentration. After calculation of the inhibition thresholds for each strain, those compounds demonstrating activity above the LmjF IPS threshold but below AUR1p threshold were selected. In addition, to avoid loss of highly potent compounds, those demonstrating >80% inhibition of the LmjF IPS dependent yeast were retained whatever the response against AUR1p dependent yeast.

As a result of this process, 4,166 compounds were progressed further for dose response analyses. Using the optimized assay conditions, these compounds were screened against the yeast dependant on either LmjF IPS or AUR1p expression at 11 concentrations between 100 μM to 1.7 nM. From these data, the −logIC50 (pIC50) molar unit values of each compound were calculated against each strain and the data plotted (Fig. 2). This allowed analyses of both potency and selectivity, with 500 compounds demonstrating potent activity against LmjF IPS dependant yeast, pIC50 ≥ 5 (IC50 ≤ 10 μM), and good selectivity when compared to AUR1p dependant yeast.

Figure 1. Generation of yeast strains for uHTS assay. Yeast lacking AUR1p completed by galactose dependent expression of AUR1p (α ade lys leu Δaur1−.pESC-LEU_AUR1) or LmjF IPS (α ade lys leu Δaur1−.pESC-LEU_LmjF IPS) grown on selective media with either glucose (A) or galactose (B). Expression of both AUR1p and LmjF IPS rescues growth of the mutant S. cerevisiae.

Figure 2. The dose response (−logIC50 M [pIC50]) of the initially identified 4,166 selective hit compounds. Yeast dependant on Leishmania IPS synthase activity (LmjF IPS) against yeast dependant on AUR1p expression. The inset shows the colour key for compound selectivity (logSI [pSI] where SI is Selectivity Index). 500 hits with a pSI > 1.50 were selected for further analyses (orange, turquoise and brown).
pSI ≥ 1.5 (logSI [pIC 50 LmjF IPCS - pIC 50 AUR1], where SI is Selectivity Index). This represented a hit rate of 0.0063% for the identification of potent and selective hits against LmjF IPCS.

These 500 compounds were subsequently clustered using a sphere exclusion algorithm. Briefly, a fingerprint for each hit was compared to every other molecule in the set and compounds demonstrating significant similarity (> 0.85 on a scale of 0–1) were sorted into clusters. Two representatives from each cluster plus all singletons, 211 compounds, were taken forward for further analyses.

Cell-based screening of identified compounds. To establish the antileishmanial activity of the 211 selected hits, they were screened against L. donovani axenic amastigotes (the mammalian, pathogenic form) in a dose response assay with 11 compound concentrations between 50 μM and 0.85 nM. The data revealed 70 compounds with a calculated pIC50 ≥ 5 (IC50 ≤ 10 μM; Fig. 3A; SI Table).

Following a screen against HepG2 cells for mammalian cytotoxicity (11 compound concentrations between 100 μM and 1.7 nM), comparing these data with that for the 70 Leishmania hits allowed identification of selective antileishmanial compounds with pSI ≥ 1 (SI ≥ 10); 49 compounds fulfilled these criteria (Fig. 3B). Following a further screen based on the predicted physicochemical properties of these 49 compounds, those compounds showing a Property Forecast Index (PFI) ≤ 8 and ≤ 4 aromatic rings were selected (Fig. 3C; SI Table 1)). These 25 compounds have relatively low molecular weights and high levels of hydrophilicity indicating they are likely to be soluble and favourable for development, and therefore were progressed to screening against L. donovani infected THP-1 macrophages, the truest representation of the pathogenic state available in vitro. The 25 compounds were screened in a dose response assay with 11 compound concentrations between 50 μM and 0.85 nM. Given the additional membrane barriers the compounds must cross to reach the intra-macrophage amastigotes, the fact that only 7 (out of 25) were completely ineffective while 10 of the remaining 18 active hits demonstrated activity at pIC50 ≥ 5 (IC50 ≤ 10 μM) was promising. Of these 10, 4 had a pSI with respect to the activity against THP-1 macrophages of ≥ 1 (Fig. 3D; SI Table 1), another had a pSI of 0.99 and was also retained. Of these 5 hits (Fig. 4), the benzazepane compounds (1 and 2) were both active in the low μM range against intra-macrophage amastigotes, pIC50 5.5 and 5.7 respectively. The dithiophene compounds (3 and 4) exhibited similar levels of activity, pIC50 5.7 and 5.9 respectively, however, the presence of the nitro group in this class was a concern given the link with toxicity issues. Similarly, the singleton (5) possesses a nitro group. In light of the above, the benzazepanes were taken forward for further analyses in a secondary screening platform.

Verification of the benzazepane mode of action. Utilizing the availability of an in-house designed and validated biochemical assay for LmjFIPCS, following re-synthesis in the free-base form (Aptuit, Verona, Italy) the two benzazepane hits were biochemically analyzed in a dose response assay (8 compound concentrations between 100 μM and 46 nM; Fig. 5). Whilst compound 2 was a modest inhibitor of LmjFIPCS (pIC50 5.2), compound 1 was a potent, sub μM, inhibitor of the enzyme (pIC50 6.8).
Figure 4. The 5 identified selective, non-toxic, hits. $-\log IC_{50}$ M ($pIC_{50}$) and logSI (pSI, where SI is Selectivity Index) values for infected macrophage assay indicated.

Figure 5. In vitro activity against LmjFIPCS. Activity of compound 1 (red; $-\log IC_{50}$ M [$pIC_{50}$] 6.8; 95% CI: 7.0–6.5) and compound 2 (blue; $pIC_{50}$ 5.2; 95% CI: 5.5–4.9) against LmjFIPCS in vitro. Values are from at least 3 independent experiments.
However, whilst compound 1 was antiprotozoal and a potent IPC synthase inhibitor, it was unclear whether the mode of action was on-target. To analyze this further, the in-house availability of a sphingolipid mutant \(L.\) major \(\Delta lcb2\) was exploited. This line\(^{23}\) lacks serine palmitoyltransferase activity, and whilst \(LmjF\) IPCS is still present it is redundant in the absence of in situ substrate. Therefore, specific inhibitors of \(LmjF\) IPCS should be inactive against \(L.\) major \(\Delta lcb2\). However, in a dose response assay, compound 1 demonstrated no significant difference in activity against both lines of \(L.\) major (pIC\(_{50}\) 5.5 for wild type and \(\Delta lcb2\): SI Fig. 1)). Therefore, it is likely that off-target activity has a role in the antileishmanial activity of this benzazepane.

Further synthetic efforts (Aptuit, Verona, Italy) led to the synthesis of compounds bearing the left-hand side of 1 and the right-hand side of 2 (compound 6) and vice versa (compound 7; Fig. 6A). Compound 6 lost activity against \(LmjF\) IPCS in the in vitro assay; however, compound 7 maintained a pIC\(_{50}\) 7.2, although it did not achieve greater than approximately 60% inhibition even at higher concentrations (Fig. 6B). In addition, and importantly, compound 7 demonstrated selectivity for \(L.\) major wild type over the \(\Delta lcb2\) mutant (pIC\(_{50}\) 5.5 versus <5; Fig. 6C). The selectivity of compound 7 for the wild type parasite over a line with a redundant \(LmjF\) IPCS (\(\Delta lcb2\)), strongly supported a target-directed mode of action. Furthermore, compound 7 demonstrated considerably greater in cellulo inhibition of IPC synthase activity than compound 1, as determined by incorporation of BODIPY® FL C\(_{6}\)-ceramide into IPC in the presence of the compounds (Fig. 7). Unfortunately, activity against intramacrophage \(L.\) donovani was lost, possibly due to a lack of compound transport to the site of action or intramacrophage stability. However, these data demonstrated that the efficacy of compound 7 against \(L.\) major is on-target inhibition of IPC synthase, thus supporting the status of \(LmjF\) IPCS as a drug target like its orthologues in the related trypanosomatid pathogen, \(T.\) brucei\(^{20}\).

**Discussion**

The identification of leads for the discovery of new drugs for the Neglected Tropical Disease leishmaniasis is a recognized priority\(^{2,6}\). To achieve this aim the development of strong industry-academia partnerships, bridging chemistry and biology, is vital\(^{23}\). Much of such collaborative efforts have employed HTS to identify antiprotozoal compounds, however high content phenotypic screening has produced a poor return of hits against \(Leishmania\) spp when
compared with the related Trypanosoma spp. This demonstrated the central importance of target-based screening for the discovery of novel antileishmanial hits which represent new chemical entities with new modes of action.

The Leishmania IPC synthase has been previously identified as a putative drug target, and more recent work has employed a modelling approach to identify coumarin derivatives as enzyme inhibitors with in vivo antileishmanial activity. To search for new classes of Leishmania IPC synthase inhibitors, in a multidisciplinary public-private partnership, we developed a novel yeast-based assay to screen the 1.8 million entity GSK compound library against the protozoan enzyme. To the best of our knowledge, this work reports the largest yeast-based, target-directed assay undertaken to date against targets from any disease state.

5 non-toxic, antileishmanial compounds were identified (a hit rate of 0.00028%), 2 pairs and one singleton. On the basis of a lack of nitro groups the benzazepanes (compounds 1 and 2) were selected to take forward. Both of these demonstrated sub μM activity against axenic L. donovani amastigotes (pIC₅₀ 6.8 and 6.4 for compounds 1 and 2 respectively; SI Table), and low μM activity against L. donovani infected macrophages (pIC₅₀ 5.5 and 5.7 for compounds 1 and 2 respectively; SI Table). The activity of these compounds in cell based assays, when compared to other recently predicted Leishmania IPC synthase inhibitors, was promising. These activity levels were similar to those seen for miltefosine, the only oral therapy for leishmaniasis but with an unclear mode of action. However, they were one or 2 orders of magnitude lower than those for amphotericin B, which functions through selective sterol binding.

Importantly, both benzazepanes demonstrated activity against the Leishmania IPC synthase in vitro, with compound 1 particularly potent (pIC₅₀ 6.8). Despite these promising findings, this compound also demonstrated off-target activities in the parasite (SI Fig 1). However, using a simple ‘mix and match’ approach a benzazepane was identified that had enhanced potency against the enzyme in vitro (pIC₅₀ 7.2) and in cellulo (Fig. 7) and demonstrated more selective, on-target antileishmanial activity (Fig. 6).

In summary, this research demonstrated the tractability of yeast-based uHTS to identify inhibitors of challenging, membranous targets such as IPC synthase, which are on-target and specific. Furthermore, the benzazepanes identified represent a new class of antileishmanial compounds with a new mode of action. Further development of these may lead to novel therapies for the Neglected Tropical Disease, leishmaniasis.

**Methods**

**Yeast cell assay.** The complete open reading frames of LmjFIPCS and AUR1p were amplified from genomic DNA using Phusion Flash PCR master mix (ThermoFisher) according to manufacturer’s guidelines. Primers were for T4 ligation of LmjFIPCS (F_BamHI cggagatctATGACGAGTCAGTGACACG and R_HindIII cccagcttTTAGTGCTACGGCAAAGCGCAG) and In-Fusion cloning (Clontech) of AUR1 (F ctcatctagggcctATGGCAAACC and R tccagctagccgccCTAAAGCCCTC). The open reading frames were subsequently cloned into the pESC-LEU vector (Agilent) and verified by sequence analyses, creating pESC-LEU_LmjFIPCS and pESC-LEU_AUR1. In this vector, expression of the open reading frame was under the control of a galactose promoter. pESC-LEU_LmjFIPCS and pESC-LEU_AUR1 were subsequently transformed into S. cerevisiae strain α ade Δlys Δleu Δaur1 – pRS316.URA ScAUR1Δ22 as previously described and selected on SD -TRP -URA -LEU agar (0-17% Bacto Yeast Nitrogen Base, 0-5% ammonium sulphate, 2% glucose, containing the appropriate nutritional supplements) at 30°C. Clones were then ‘cured’ of the pRS316-URA plasmid by selection on SGR -TRP –LEU + FOA agar (0-17% Bacto Yeast Nitrogen Base, 0-5% ammonium sulphate, 0-1%
galactose, 1% raffinose, 0.1% 5-Fluoroorotic Acid Monohydrate (FOA) containing the appropriate nutritional supplements) at 30 °C, creating α ade -lys -leu -Δaur1 -pESC-LEU_LmjFIPCS and α ade -lys -leu -Δaur1 -pESC-LEU_AUR1. Following validation by PCR and propagation in SGR -TRP -LEU, frozen stocks of both yeast lines were created (OD<sub>600</sub> = 10). 10 μM FDGlu and α ade -lys -leu -Δaur1 -pESC-LEU_LmjFIPCS or α ade -lys -leu -Δaur1 -pESC-LEU_AUR1 (defrosted on ice) at OD<sub>600</sub> = 0.0625 in SGR -TRP -LEU were prepared and dispensed into 1536-well plates (5 μl per well; ThermoFisher) with appropriate concentrations of compound and controls (vehicle or 1 mM cycloheximide). Volumes were dispensed using a Multidrop Combi Reagent Dispenser, a Multidrop Combi nL or a Labcyte Echo® liquid handler (ThermoFisher). Plates were then incubated, in duplicate, at 30 °C for 25 hours prior to the addition of phosphate buffer (0.25 M, pH 7.0, 5 μl per well) and fluorescence was read at Ex480/Em540 using a ViewLux ultraHTS Microplate Imager (PerkinElmer). Data were analyzed using GSK-developed Statistical Online Data Analysis Software (SODA) and ActivityBase (IDBS).

**Leishmania axenic cell assays.** As previously described<sup>18</sup>, *L. donovani* (MHOM/SD/62/1S-CL2D, LdBOB) axenic amastigotes in growth medium (6 μl) were added to a final concentration of 5.0 × 10<sup>3</sup> ml<sup>−1</sup> with compounds and controls (vehicle and assay media alone, with the robustness of the platform indicated by Z’ values > 0.5)<sup>29</sup> in 1536-well plates (Greiner Bio-One FLUOTRAC). The plates, in at least triplicate, were incubated at 37 °C for 72 hours under 5% CO<sub>2</sub>. Resazurin solution was added, the plates were incubated at room temperature for 4 hours and then the fluorescence read at Ex528/Em590, using an EnVision Multilabel plate reader (PerkinElmer). Volumes were dispensed and data analyzed as above. From a previously described protocol<sup>22</sup>, *L. major* (MHOM/IL/81/Friedlin, wild type and ΔLCB2 mutant)<sup>24</sup> were added to 96-well plates (Corning) at 1 × 10<sup>4</sup> ml<sup>−1</sup> in growth media with appropriate concentrations of compounds and controls (vehicle and assay media alone, with the robustness of the platform indicated by Z’ values > 0.5)<sup>29</sup> in 100μl. Following incubation at 26 °C for 72 hours, alamarBlue (ThermoFisher) was added, the plates were incubated at 26 °C for 4 or 8 hours and fluorescence was read at Ex540/Em600, using a FLx800 Fluorescence Microplate Reader and Gen5 1.08 Data Analysis Software (BioTek). Analyses were carried out using GraphPad Prism 7.

**Mammalian cell assay.** HepG2 cells in media were added (25 μl) at final concentration of 1.2 × 10<sup>5</sup> ml<sup>−1</sup> to 384-well plates (Greiner Bio-One FLUOTRAC) and incubated with compounds and controls (vehicle and cycloheximide) at 37 °C for 48 hours under 5% CO<sub>2</sub> and 80% relative humidity. The plates, in at least duplicate, were incubated at room temperature for 30 minutes prior to the addition of CellTiter-Glo (Promega), followed by incubation at room temperature for 10 minutes and measurement of the luminescent output using a ViewLux ultraHTS Microplate Imager (PerkinElmer). Volumes were dispensed using a Multidrop Combi Reagent Dispenser, a Multidrop Combi nL or a Labcyte Echo liquid handler (ThermoFisher). Data were analyzed using GSK-developed Statistical Online Data Analysis Software (SODA) and ActivityBase (IDBS).

**Intramacrophage Leishmania donovani assay.** This protocol was based on that previously described<sup>18</sup>. In brief, THP-1 (human monocytic leukemia) at 6 × 10<sup>4</sup> ml<sup>−1</sup> were infected with eGFP expressing *L. donovani* LdBOB amastigotes at 6.0 × 10<sup>7</sup> ml<sup>−1</sup> and incubated overnight at 37 °C. Following washing, infected cells were trypsinized and harvested before dispensation into a sterile 384-well plate, at 6.0 × 10<sup>4</sup> ml<sup>−1</sup> in 50 μl post-differentiation growth medium with compounds and controls (vehicle and amphotericin B, with the robustness of the platform indicated by Z’ values > 0.5)<sup>29</sup>. The plates, in at least duplicate, were incubated at 37 °C for 96 hours under 5% CO<sub>2</sub>. After fixation with 4% (v/v) formaldehyde in PBS at room temperature for 30 minutes and washing, cells were stained with DAPI (Sigma-Aldrich) at room temperature for 30 minutes, washed, and imaged at Ex405/Em460 and Ex488/Em509 using the Opera® High Content Screening System (PerkinElmer). Volumes were dispensed as above. Images were analyzed using the automated Acapella High Content Imaging and Analysis Software (PerkinElmer).

**Biochemical assay.** Micromolar material was prepared and CHAPS washed from α ade -lys -leu -Δaur1 -pESC-LEU_LmjFIPCS as previously described<sup>19,22</sup>. Briefly, each compound at the desired concentration was incubated, in at least triplicate, in 96-well plates (Corning) in phosphate buffer (71.4 mM, pH 7.0) with PI (100 μM, final concentration, Avanti Polar Lipids), CHAPS (600 μM, Sigma) and CHAPS-washed microsomal membranes (0.6 µ enzyme per reaction) for 15 minutes at 30 °C. NBD-C<sub>2</sub>-ceramide (5 μM; ThermoFisher) was added, final volume of 40 μl per well, and the plates incubated at 30 °C for a further 25 minutes. Following quenching with 200 μl methanol per well, the reaction product was separated using exchange chromatography in 96-well filter plates (Millipore)<sup>19,22</sup> and the fluorescence measured at Ex460/Em540 using a FluoroMax Fluorescence Microplate Reader and Gen5 1.08 Data Analysis Software (BioTek). Analyses were carried out using GraphPad Prism 7.

**In cellulo IPC synthase assay.** The *L. major* Δlcb2 mutant<sup>24</sup>, maintained in Schneider’s media (Sigma-Aldrich) with 15% sera (ThermoFisher), was washed and 200 μl at 1 × 10<sup>4</sup> ml<sup>−1</sup>, incubated in serum free media for 60 minutes at 26 °C before the addition of 10μM of the compounds, in at least triplicate, and further incubation for 18 hours. Subsequently, the reaction was initiated by the addition of BODIPY FL C<sub>2</sub>-ceramide complexed to BSA (1.25 μl of 0.5 mM, ThermoFisher). Following further incubation at 26 °C for 1 hour, the lipids were phase separated in chloroform:methanol:water (10:10:3, 200 μl) and fractionated on HTPLC plates, as previously<sup>18</sup>. The fluorescence was read at Ex475/Em520 using a Fuji FLA-3000 plate reader and AIDA Image Analyser software (version 3.52).
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Author Contributions
J.L.N. and J.G.M. constructed, validated and utilised the yeast assay; E.A., F. de D.-A. and G.C. managed all aspects of the screening process; S.G.-Del V., M.M. and J.M.F. oversaw project management; J.L.N., J.G.M. and J.C. analysed data and constructed figures; J.J.M., P.G.S and P.W.D. conceived, directly managed the project and analysed data; P.W.D. wrote the manuscript and constructed figures.

Additional Information
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