The Susceptibility of Trypanosomatid Pathogens to PI3/mTOR Kinase Inhibitors Affords a New Opportunity for Drug Repurposing

Rosario Diaz-Gonzalez¹, F. Matthew Kuhlmann², Cristina Galan-Rodriguez³, Luciana Madeira da Silva⁴, Manuel Saldivia¹, Caitlin E. Karver⁵, Ana Rodriguez³, Stephen M. Beverley⁴, Miguel Navarro¹, Michael P. Pollastri*⁴

¹Instituto de Parasitología y Biomedicina “López-Neyra” Consejo Superior de Investigaciones Científicas, Granada, Spain, ²Department of Medicine-Division of Infectious Diseases, Washington University School of Medicine, St. Louis, Missouri, United States of America, ³Division of Medical Parasitology, Department of Microbiology, New York University School of Medicine, New York, New York, United States of America, ⁴Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri, United States of America, ⁵Department of Chemistry and Chemical Biology, Northeastern University, Boston, Massachusetts, United States of America

Abstract

**Background:** Target repurposing utilizes knowledge of “druggable” targets obtained in one organism and exploits this information to pursue new potential drug targets in other organisms. Here we describe such studies to evaluate whether inhibitors targeting the kinase domain of the mammalian Target of Rapamycin (mTOR) and human phosphoinositide-3-kinases (PI3Ks) show promise against the kinetoplastid parasites Trypanosoma brucei, T. cruzi, Leishmania major, and L. donovani. The genomes of trypanosomatids encode at least 12 proteins belonging to the PI3K protein superfamily, some of which are unique to parasites. Moreover, the shared PI3Ks differ greatly in sequence from those of the human host, thereby providing opportunities for selective inhibition.

**Methodology/Principal Findings:** We focused on 8 inhibitors targeting mTOR and/or PI3Ks selected from various stages of pre-clinical and clinical development, and tested them against *in vitro* parasite cultures and *in vivo* models of infection. Several inhibitors showed micromolar or better efficacy against these organisms in culture. One compound, NVP-BEZ235, displayed sub-nanomolar potency, efficacy against cultured parasites, and an ability to clear parasitemia in an animal model of T. brucei rhodesiense infection.

**Conclusions/Significance:** These studies strongly suggest that mammalian PI3/mTOR kinase inhibitors are a productive starting point for anti-trypanosomal drug discovery. Our data suggest that NVP-BEZ235, an advanced clinical candidate against solid tumors, merits further investigation as an agent for treating African sleeping sickness.


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The discovery of new therapeutic agents is expensive and time consuming, and various strategies have been implemented in order to mitigate costs and speed drug discovery [7]. While the pharmaceutical industry frequently begins drug discovery programs with high-throughput screening and extended medicinal chemistry research programs, this paradigm remains unaffordable for most not-for-profit endeavors to implement. Therefore, the approach of “target repurposing” is frequently employed, where molecular targets in parasites are matched with homologous human targets that have been previously pursued for drug discovery [8,9,10,11]. In the best case, drugs that are selective for these human targets will have been carried into human clinical studies, strongly suggesting that the homologous parasite target is likely “druggable” [12], that is, that compounds can be designed to inhibit the target that are safe and orally bioavailable.
Author Summary

In our study we describe the potency of established phosphoinositide-3-kinase (PI3K) and mammalian Target of Rapamycin (mTOR) kinase inhibitors against three trypanosomatid parasites: Trypanosoma brucei, T. cruzi, and Leishmania sp., which are the causative agents for African sleeping sickness, Chagas disease, and leishmaniasis, respectively. We noted that these parasites and humans express similar kinase enzymes. Since these similar human targets have been pursued by the drug industry for many years in the discovery of cellular growth and proliferation inhibitors, compounds developed as human anti-cancer agents should also have effect on inhibiting growth and proliferation of the parasites. With that in mind, we selected eight established PI3K and mTOR inhibitors for profiling against these pathogens. Among these inhibitors is an advanced clinical candidate against cancer, NVP-BEZ235, which we demonstrate to be a highly potent trypanocide in parasite cultures, and in a mouse model of T. brucei infection. Additionally, we describe observations of these inhibitors’ effects on parasite growth and other cellular characteristics.

With an eye towards target repurposing for anti-trypanosomal drug discovery, we have identified the trypanosomal phosphoinositide 3-kinases (PI3Ks) as a promising class of targets for pursuit. In humans, inhibition of members of the PI3K family has attracted significant interest as targets in the discovery of new anticancer and anti-inflammatory agents [13,14,15]. This kinase family provides critical control of cell growth and metabolism, and is comprised of three classes (I-III), as determined by structure, regulation, and substrate specificity. The Target of Rapamycin (TOR) kinase (a member of the PI3K-related kinase (PKK) subfamily) has received particular interest due to its central role in fundamental processes such as growth, cell shape and autophagy. The TOR kinases were first identified through inhibition studies with the natural product rapamycin and related compounds. This inhibition is now known to be mediated through interactions of the TOR FKBP12-rapamycin-binding (FRB) domain with the rapamycin-binding protein FKBP12 [16,17]. More recently, inhibitors targeting the mammalian TOR (mTOR) kinase domain have been developed [18,19,20,21,22,23]. In addition, significant effort has been employed to discover inhibitors targeting specific PI3K family members [24].

Thus far, while some agents show selectivity for mTOR or for various specific PI3Ks, selectivity is rarely absolute. Many inhibitors show broad activity against a spectrum of PI3K or TOR family members. Nonetheless, both selective mTOR and these so-called “mixed” PI3K inhibitor classes have shown promise as cancer therapeutics, suggesting that absolute specificity may not be required for therapeutic efficacy [25,26]. Some key examples of these mTOR-selective and mixed inhibitors are shown in Table 1 and Figure 1.

Database mining of trypanosomatid genomes has revealed the presence of at least 12 proteins belonging to the PI3K protein superfamily (PFAM PF00454), many of which are unique to the parasites. Notably orthologous proteins are highly divergent from those of the human host. These include predicted kinases related to the eukaryotic class I and II PI3Ks, PI4Ks, and PIKKs including TOR, ATM and ATR ([27,28], and data not shown). Where tested, PI3Ks appear to be essential for viability and/or virulence in trypanosomatids. Two PIK subfamily members have been examined in T. brucei. The trypanosome Class III PI3K TbVps34 has an essential function in membrane trafficking and in Golgi segregation during cell division [29]. These authors suggested that, similar to yeast, T. brucei possesses only one genuine PI3K. TbPI3Kb is also an essential protein in T. brucei, required for maintenance of Golgi structure, protein trafficking, and cytokinesis [29]. Trypanosomatids possess four distinct genes belonging to the TOR family, in contrast to mammals, which possess a single mTOR protein [30,31,32,33]. TORs act in concert with other proteins in complexes referred to as TORCs, which have different protein subunit compositions, and cellular functions [34].

In T. brucei, the two conserved signaling complexes, TORC1 and TORC2, whose functions appear analogous to that described in mammalian or yeast TORCs, mediate the essential functions of TOR1 and TOR2 for cell growth [33,35]. While TbTORC1 regulates protein synthesis, cell cycle progression and autophagy, TbTORC2 plays a key role in maintaining the polarization of the actin cytoskeleton, which is required for the proper functioning of endocytic processes, cell division, and cytokinesis [30,36]. Correspondingly, TOR1 and TOR2 are essential genes in Leishmania major [31]. Recent work has characterized a third TOR protein, TOR3, in Leishmania major and T. brucei, that is implicated in the formation of acidocalcisomes and participation in stress response [31,32]. A fourth TOR in T. brucei and Leishmania (TOR4) lacks the FRB domain responsible for binding rapamycin-binding proteins, yet possesses all other characteristic domains of TOR kinases [30,31].

The essentiality of several PIKs and TOR1 and TOR2 and the requirement for TOR3 for virulence in both trypanosomes and Leishmania provide genetic validation of these essential kinases as potential drug targets. Since rapamycin analogs are relatively modest inhibitors of trypanosomatid TORs and/or parasite growth [30,31,37] and difficult to synthesize, we focused in this work on kinase domain inhibitors under development. As these kinase domain inhibitors are generally more drug-like, soluble, and synthetically accessible than rapamycin analogs, we anticipate these properties could facilitate future optimization efforts.

Materials and Methods

Ethics statement

The animal experimental protocol (2010102/1) used for African trypanosome studies was reviewed and approved by the Ethical Committee IPBLX-CSIC of the Spanish Council of Scientific Research (CSIC). For T. cruzi, animal studies were approved by the Institutional Animal Care and Use Committee of New York University School of Medicine (protocol #81213), which is fully accredited by the Association For Assessment and Accreditation Of Laboratory Animal Care International (AAALAC). For L. major, animal studies were approved by the Animal Studies Committee at Washington University (protocol #20090006) in accordance with the Office of Laboratory Animal Welfare’s guidelines and AAALAC.

Inhibitor compounds

Inhibitor compounds were received from commercial vendors and used as received. PI-130, NVP-BEZ235, Ku-0063794, Pp242, and WYE-354 were obtained from Chemdea, Inc. ( Ridgewood, NJ). LY294002, LY303511, and Compound 401 were obtained from Tocris Biosciences (Ellisville, MO).

Potency assessment against T. brucei

Assays were performed using the strain of T. brucei brucei Lister 427 adapted to the laboratory, and the human-infective strain T. b.
Both strains were grown and tested as bloodstream forms. To establish the EC50, cultures of *Trypanosoma brucei* and *T. b. rhodesiense* were treated with two-fold increasing concentrations of compounds (with similar DMSO increasing concentration as control). We also utilized *T. b. gambiense* strain Eliane MHOM/CI/52/ITMAP 2188, and another *T. b. brucei*, strain 927/4 GUTat10.1 [38]. Cell populations were measured at 72 hours with an Infinite F200 microplate reader (Tecan Austria GmbH, Austria); the determination of cell viability was carried out by the established colorimetric technique AlamarBlue® with modifications, a 96-well plate format spectrophotometric assay which measures the ability of living cells to reduce resazurin [39,40]. Data obtained with *T. b. brucei* Lister 427 were confirmed by manual counting in a

**Table 1.** Selectivity profile of the selected inhibitors against human enzymes.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibition of Cell Growth</th>
<th>mTOR</th>
<th>PI3K</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EC50 (μM)</td>
<td>IC50 (μM)</td>
<td>IC50 (μM)</td>
<td>IC50 (μM)</td>
</tr>
<tr>
<td>NVP-BEZ235</td>
<td>0.05</td>
<td>&lt;0.01</td>
<td>0.004</td>
<td>0.075</td>
</tr>
<tr>
<td>PI-103</td>
<td>0.5</td>
<td>0.02</td>
<td>0.0036</td>
<td>0.003</td>
</tr>
<tr>
<td>LY294002</td>
<td>5</td>
<td>0.73</td>
<td>0.31</td>
<td>1.06</td>
</tr>
<tr>
<td>LY303511</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Compound 401</td>
<td>1</td>
<td>5.3</td>
<td>&gt;100</td>
<td></td>
</tr>
<tr>
<td>Pp242</td>
<td>0.04</td>
<td>0.008</td>
<td>2.8</td>
<td>2.2</td>
</tr>
<tr>
<td>WYE-354</td>
<td>0.03</td>
<td>0.004</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Ku-63794</td>
<td>0.03</td>
<td>0.01</td>
<td>8.9</td>
<td>&gt;30</td>
</tr>
</tbody>
</table>

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**Figure 1.** Inhibitors selected for this study. These include that are (a) selective for the mTOR kinase domain, and (b) inhibit both mTOR and human PI3Ks.

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Neubauer chamber for a direct microscopic examination to rule out multinucleated phenotypes that could mask the colorimetric assays, as well as the subtraction of solvent background to dismiss a potential solvent-derived fluorescence. Pentamidine was used as drug control for potency comparison, and T. b. brucei Lister 927 strain was included in our experiments to evaluate the adaptation to medium for the different strains as a variable condition.

Analysis of morphological and cell cycle alterations from compound treatment in T. brucei

Flow cytometry was used to assess cell size and DNA content, to reveal a G1 or G2 arrest and multinucleated cells. Briefly, bloodstream cells of T. brucei brucei Lister 427 strain in early log phase culture were treated with high dose (1 μM for PI-103, 2 μM for WYE-354 and Pp242 and 100 nM for NVP-BEZ235) of compounds for 16 hours, when the cells were pelleted and washed to remove all traces of drug. After permeabilization with 1 μL saponin (0.5 mg/mL final concentration), the culture was RNase treated for 30 minutes (10 μg/mL final concentration) and stained with 20 μg/mL propidium iodide immediately before its acquisition in a FACScan cytometer. Cells incubated with equivalent concentration of drug solvent (DMSO) were included in each experiment as control population.

Potency assessment against T. cruzi

T. cruzi trypomastigotes from the Tulahuen strain stably expressing the β-galactosidase gene [41] were obtained from the supernatant of infected cultures of LLC-MK2 cells harvested between days 5 and 7. To remove amastigotes, trypomastigotes were allowed to swim out of the pellet of samples that had been centrifuged for 7 min at 2500 rpm.

For measurement of intracellular replication, 5×10⁴ NIH/3T3 cells and 5×10⁴ trypomastigotes per well were seeded in 96-well plates in DMEM supplemented with 2% FBS and Pen-Strep-Glut. DMEM did not contain phenol red to avoid interference with the assay absorbance readings at 590 nm. After 3 hours, compounds were added to a final volume of 200 μL/well at the indicated concentrations and mixed by pipetting. A 4 μM Amphotericin B solution (Sigma-Aldrich) was used as positive control. After 4 days of incubation at 37°C 5% CO₂, 50 μL of PBS containing 0.5% of NP40 and 100 μM chlorophenol red-β-galactoside (CPRG) (Fluka) were added to each well. Plates were incubated at 37°C for 4 hours and absorbance was read at 590 nm.

For evaluation of extracellular survival, free trypomastigotes were rinsed once and placed in 96-well plates at 100,000/well with the compounds in a final volume of 200 μL of DMEM (without phenol red) supplemented with 2% FBS, Pen-Strep-Glut and 100 μM CPRG. Plates were incubated for 24 h at 37°C and absorbance was read at 590 nm.

Potency Assessment against Leishmania

Leishmania major strain FV1 (MHOM/IL/80/Friedlin) was grown in M199 media [42]. Leishmania donovani strain LeBob (MHOM/SD/62/1S-CL2D) were grown in modified M199 media as promastigotes (26°C) [43]. Amastigote specific media (37°C) was used for growth and differentiation of amastigotes [43]. L. donovani axenic amastigotes were passed once following differentiation prior to use. Cells were enumerated using a Coulter Counter (BD Biosciences); as amastigotes tend to grow in clumps, L. donovani axenic axamastigotes were passed gently through a blunt 27-gauge needle prior to counting. For determination of EC₅₀ values, log phase cells were inoculated at concentration of 10⁷/ml into appropriate media with compounds as indicated, and counted when the controls lacking drug had reached late logarithmic phase. The EC₅₀ is defined as the concentration of drug inhibiting 50% of control growth, and was calculated by linear regression analysis using SigmaPlot 2000.

Cell size and DNA content analysis in Leishmania

L. major log phase promastigotes were inoculated at a concentration of 10⁶ cells/ml into media with compounds as indicated, and incubated overnight with varying drug concentrations to assess cell size and DNA content. For cell size, forward scatter of live promastigotes was measured by a FACS flow cytometer (Becton Dickinson), utilizing dye exclusion with 5 μg/ml propidium iodide (PI) to gate for live cells. DNA content was determined by flow cytometry using fixed and permeabilized L. major stained with PI as previously described [44,45], but reducing the incubation time with PI and RNase A from 1 hour to 30 minutes. Histogram analysis was performed using CellQuest 3.1 software (BD Bioscence).

Drug dosage for in vivo experiments

The targeted dosage of inhibitors was determined based on the pharmacokinetic studies disclosed by Maíra, et al. [46]. Our goal was to test NVP-BEZ235 in the animal models at the highest dose achievable without inducing toxicity. For L. major, 12.5 mg/kg orally was the highest tolerable dose while 30 mg/kg intraperitoneally (ip) was used for the T. cruzi infections. A lower dose was initially used in T. brucei, 5 or 10 mg/kg intraperitoneally.

In vivo drug evaluation in T. brucei

Female Balb/C mice (Jackson Laboratories, Bar Harbor, ME) were infected with 10⁸ cells of an early log phase culture of T. b. rhodesiense EATRO3; 72 hours after infection the mice were arbitrarily separated into three independent groups, daily treated with 5 or 10 mg/kg NVP-BEZ235, 20 mg/kg pentamidine, or DMSO, via intraperitoneal injection for four days. The parasitemia was checked at days 3, 5, 7, 11 and 14 post-infection in alive mice: in those cases the parasitemia was too low to detect by Neubauer chamber count, the extracted blood was incubated in a 24-well plate with HMI-9 medium supplemented with 20% SBFi at 37°C with 5% CO₂, and positive wells were confirmed by direct visualization of parasites. 22% of parasitized animals were sacrificed for analysis using SigmaPlot 2000.

In vivo drug evaluation in T. cruzi

Balb/c mice were inoculated intraperitoneally with 10⁷ trypomastigotes from T. cruzi Y strain expressing firefly luciferase (kindly provided by Dr. Barbara Burleigh, Harvard University). On day 7 post infection, mice were anesthetized with ketamine/xylazine and injected with 3 mg of D-Luciferin Potassium Salt (Gold Biotechnology) at 20 mg/ml in PBS and imaged in the IVIS Lumina II (Caliper Life Sciences). On day 8, groups of five mice were injected intraperitoneally with either 30 mg/kg of NVP-BEZ235 in DMSO or only DMSO, as control. Mice were treated for 5 days and imaged again on day 13. Data is expressed as the ratio between luciferase units in day 13 versus day 7 to determine the progression of infection with and without drug treatment.

In vivo drug evaluation in L. major

Mice were infected with luciferase expressing L. major (LmFV1LucTK-1) and analyzed by bioluminescent imaging as described [47]. Balb/c mice were infected with 10⁷ L. major...
metacyclic stage parasites purified by gradient centrifugation [48]. Luminescence was measured using an IVIS 100 instrument and analyzed with Living Image software version 2.60. NVP-BEZ235 was resuspended in DMSO and applied at 12.5 µg/kg/day by oral gavage for 10 days, with treatment starting day 17 post infection. At this dose the mice showed significant weight loss, suggesting that this dosage was the highest practicable, as dosing intraperitoneally at 25 µg/kg/day was lethal.

List of accession numbers

The following trypanosomatid enzymes are discussed in the text: LmjF36.6320 (LmjTOR1), LmjF34.4530 (LmjTOR2), LmjF34.3940 (LmjTOR3), LmjF20.1120 (LmjTOR4), Tb927.8.6210 (TbVps34), Tb927.4.1140 (TbPHK beta), Tb927.5.4020 (TbPHK alpha), Tb927.10.0420 (TbTOR1), Tb927.4.420 (TbTOR2), Tb927.4.800 (TbTOR3) Tb927.1.1930 (TbTOR4), Tb1.01.6300 (TbATR), Tb927.2.2260 (TbATM).

Results

Compound selection

We selected eight commercially-available compounds (Figure 1, Table 1) to profile for activity against Trypanosoma brucei, T. cruzi and two species of Leishmania, cutaneous L. major and visceral L. donovani. In order to identify potential inhibitors of trypanosoma TORs or PI3Ks, we selected a range of compounds with varied potencies and selectivities against mTOR/PI3K. In mammalian cells, compounds Ku-0063794 [22,23], Pp242 [19], and WYE-354 [49] inhibit the kinase domain of mTOR selectively with low nanomolar IC50 values. LY294002 is a mixed inhibitor targeting both mTOR/PI3K [50], and many analogs have been made (including LY303511, which inhibits mTOR-dependent and independent pathways, but does not inhibit PI3Ks [51,52]). PI-103 inhibits PI3Ks with high potency and mTOR with a reported 20 nM IC50 [53,54,55]. Compound 401, a compound structurally related to LY303511, inhibits mTOR and cellular growth at low micromolar concentrations [56], while NVP-BEZ235 inhibits both PI3Ks and mTOR with sub-nanomolar IC50 values [57,58].

In vitro testing of inhibitors

We first tested these compounds against parasites grown in vitro. For T. brucei and Leishmania donovani, it is possible to cultivate free parasites in vitro as the infective stage forms: bloodstream form (BSF) for T. brucei, and axenic amastigotes for L. donovani. Compounds were also tested against L. major promastigotes (the stage carried normally by the insect vector). To study infective forms of T. cruzi, compounds were added simultaneously with drug at an EC50 (the effective concentration at which 50% of the parasites are inhibited) produced a combination of effects on the cell cycle progression at 0.1 µM, including the appearance of zoids (anucleated cells) [60] and multinucleated cells. This relatively small but noticeable reduction in cell size.
Effect of PI3K/mTOR Inhibitors on Trypanosomatids

Table 2. Summary of potency data of mTOR/PI3K inhibitors against trypanosomatid cultures.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Leishmania sp.</th>
<th>Trypanosoma sp.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L. major</td>
<td>L. donovani</td>
</tr>
<tr>
<td>PI-103</td>
<td>0.32±0.16</td>
<td>1.05±0.28</td>
</tr>
<tr>
<td>NVP-BEZ235</td>
<td>0.11±0.05</td>
<td>0.14±0.08</td>
</tr>
<tr>
<td>LY294002</td>
<td>&gt;25</td>
<td>-</td>
</tr>
<tr>
<td>Compd 401</td>
<td>&gt;25</td>
<td>-</td>
</tr>
<tr>
<td>Pp242</td>
<td>2.4±0.8</td>
<td>0.42±0.03</td>
</tr>
<tr>
<td>WYE-354</td>
<td>4.1±0.3</td>
<td>5.95±0.84</td>
</tr>
<tr>
<td>Ku-63794</td>
<td>&gt;25</td>
<td>-</td>
</tr>
</tbody>
</table>

*promastigotes, average of three replicates; 
auxenic amastigotes, average of three replicates; 
trypomastigotes, average of three replicates, within ±10.2%; 
bloodstream form, average of three replicates.

*p<0.05 for L. major vs. L. donovani promastigotes; 
#p<0.05 for L. major promastigotes vs. L. donovani promastigotes or amastigotes.

Effective concentration (EC50) values are shown in micromolar concentrations except as noted.

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In vivo tests

We chose the most active inhibitor, NVP-BEZ235, for testing in appropriate animal models of T. brucei rhodesiense, T. cruzi, and L. major infection. Using the highest tolerable doses appropriate for each infection model, no efficacy was observed against either T. cruzi (30 mg/kg, 5 days, intraperitoneal) or L. major (12.5 mg/kg/day, 10 days, oral gavage) (data not shown). Weight loss was observed in drug-treated mice infected with L. major and higher drug doses were lethal.

In contrast, a marked decrease in parasitemia was observed by intraperitoneal dosage (5 or 10 mg/kg) of NVP-BEZ235 in T. brucei rhodesiense infected mice. Drug was administered once per day, for four days. A dramatic decrease in parasitemia was observed within two days, below the detection limit of 10^3 parasites/mL. All mice in the untreated group died the 6th day post-inoculation, while the mean survival day (MSD) for animals treated with 5 mg/kg of NVP-BEZ235 was extended to 10.8 (±2.4) days. The MSD of mice treated with 10 mg/kg increased to 13.4 (±3.3) days, doubling the survival of the control group (Figure 4). In comparison, parasitemia was below detectable limits after two days of treatment with pentamidine (20 mg/kg, ip [61]), and parasite counts remained below these limits for 30 days past dosing (data not shown).

Discussion

The intent of these experiments was to identify promising small molecules that could represent a starting point for further medicinal chemistry optimization and a better understanding of molecular pharmacology. Noting the functional and structural homology between TOR and PI3Ks in humans and trypanosomatids and encouraged by the remarkable growth inhibitory phenotype resulting from depletion of TOR in T. brucei [30] and L. major [31], we identified and procured eight established inhibitors for assessment against parasite cultures. Several of these inhibitors inhibited parasite growth in all species/strains tested, and one, NVP-BEZ235, reduced parasitemia in an animal model of T. brucei rhodesiense infection, significantly extending survival. NVP-BEZ235 showed very potent inhibition of T. brucei growth, with a phenotype similar to that seen previously in genetic studies of TOR [30] and TbPep34 [29]. A modest treatment regime (10 mg/kg, for four days) was able to eliminate 80% of the parasites in T. b. rhodesiense infections. The in vitro potency (179 pM EC50) observed against T. b. gambiense was exceedingly high, at a level rarely seen against these protozoan parasites. Given these encouraging results, we believe that additional studies of NVP-BEZ235 are warranted in the future, with the goal to determine whether an NVP-BEZ235 dosing regimen may be formulated able to achieve therapeutically useful effects.

The reason for the difference in potency between T. b. rhodesiense and gambiense, and between T. b. brucei Lister 427 and 927, is not known at this time. (Table 3). T. b. gambiense, with a lower generation rate and poorer adaptation to culture than T. b. rhodesiense, may be more affected by an antiproliferative drug as NVP-BEZ235. The potency of this compound and the complex cell cycle phenotype observed suggest that the compound likely has a number of molecular targets in T. brucei, perhaps affecting other essential cellular functions besides cell proliferation.

T. cruzi was relatively insensitive to the inhibitors compared to T. brucei. This may arise from the fact that T. cruzi trypomastigotes, the form of the parasite that proliferates in the human, only replicates in the intracellular environment. As a consequence, compounds need to cross the plasma membrane of the host cell to have access to T. cruzi, while T. brucei is directly accessible to the drugs in the bloodstream. When NVP-BEZ235 was tested against free, non-replicating T. cruzi trypomastigotes, it was inactive, while it induced lysis of intracellular T. cruzi amastigotes. This raises the possibility that this compound acts either specifically against the amastigote stage, or through effects on host cell PI3Ks, or some combination. The involvement of host cell PI3K and mTOR pathways in immune evasion has been recently reported for L. donovani [62].
Leishmania showed a range of sensitivities to the panel of inhibitors, with the most potent compounds active at sub-micromolar concentrations. This may be compared to the efficacy of current front line anti-leishmanial agents, whose potencies when measured by methods similar to those described here range from 30 nM for amphotericin B to 15 μM for antimonial based compounds [63].

The EC_{50} values for the compounds tested here were similar for L. major promastigotes, L. donovani promastigotes and L. donovani axenic amastigotes, suggesting that preliminary screening against a
single form could be sufficient in the future. However, despite its potency against L. donovani axenic amastigotes, high doses of NVP-BEZ235 against L. donovani infections of mice showed no therapeutic effect (data not shown). As discussed above for T. cruzi, the lack of efficacy in the animal model of L. major could reflect a similar need for the drug to traffic to the phagolysosomal compartment where Leishmania reside. The 12.5 mg/kg oral dosing regimen tested here resulted in severe weight loss (not shown) suggesting attempts to treat with higher doses of NVP-BEZ235 would result in significant toxicity.

Current data suggest there are at least 12 members of the PI3K protein superfamily, for which the phenotypic effects of inhibition or genetic deletion in T. brucei or Leishmania are known for only five. Thus, it is difficult to assess from our studies what the likely cellular target may be. However, with an eye towards initial identification of specific targets potentially involved in the activity of NVP-BEZ235, we note a similar cell cycle phenotype that Hall et al. reported upon RNAi knockdown of TbVsp34 [29], including the appearance of zoids, multinucleated cells and reduction of G1 and G2 (Figure 3B). Barquilla et al. also showed the same phenotype after RNAi of TOR2 [36].

In trypanosomes and mammals, TORC1 inhibition is known to result in G1 arrest and decreased cell size, while TORC2 results in G2 arrest and increased cell size [30,34,64,65,66,67]. In both T. brucei and Leishmania PI-103 resulted in G1 arrest and cell size reduction, while NVP-BEZ235 resulted in aberrant cell cycle and multiple cell sizes (Figure 3). WYE-354 also resulted in G1 arrest/cell size decrease in Leishmania. In contrast, while Pp242 showed G1 arrest/cell size decrease in trypanosomes, this was only found at lower drug concentrations in L. major, and at higher drug concentrations G2 arrest and increased cell size was observed instead. Thus, while the effects of specific inhibitors on trypanosomatids may resemble those seen against mammalian cells targeting specific TOR or PI3K targets, future studies will be required to more definitely establish the true mode(s) of action against the individual parasite species, which may differ.

It appears that the mTOR/PI3K inhibitors display generally superior activity against trypanosomatid growth over mTOR-selective inhibitors. This may be suggestive of the effect being mediated via inhibition of multiple trypanosomal PI3Ks, including...
PKKs such as TOR. With that in mind, efforts to identify the mechanism of action of these mTOR inhibitors in trypanosomatids will direct further medicinal chemistry efforts. Despite the lack of certainty of the mechanism of action of these compounds, the results we report in this work provide a validation for the repurposing approach as an efficient approach to identification of compounds that can potentially be effective in parasite killing.

Conclusions
In summary, by application of the target repurposing approach, we have identified a series of established mTOR and mTOR/PI3K inhibitors that display a range of activity against the trypanosomatid parasites T. brucei, T. cruzi, and Leishmania. These compounds provide a promising starting point for discovery of new drugs for trypanosomal infections. While additional study is needed to determine the exact mechanism of action of these agents, these results indicate promising inroads to a new class of therapeutics. Encouragingly, the most potent and effective compound identified in these studies, NVP-BEZ235, is in clinical testing as an anticancer agent, and, if approved for this primary indication, may also warrant exploration as an anti-trypanosomal agent.

Author Contributions
Conceived and designed the experiments: SMB RD-G CEK FMK MN MPP CG-R AR LMS. Performed the experiments: RD-G CEK FMK CG-R LMS MS. Analyzed the data: RD-G CEK FMK CG-R LMS MS. Wrote the paper: SMB MS MPP AR.

References

Figure 4. Trypanocidal activity of NVP-BEZ235 in an acute mouse infection model. Three independent groups (n = 5 per dose group) were infected with T. b. rhodesiense, and treated with DMSO, 5 mg/kg or 10 mg/kg of NVP-BEZ235, intraperitoneally, once a day. The arrow indicates the drug dosing schedule. The mean parasitemia for each group is represented for each day up to the death of all mice in a group. The mean survival day (MSD) is labeled in the graphic with daggers. doi:10.1371/journal.pntd.0001297.g004


Effect of PI3K/mTOR Inhibitors on Trypanosomatids