Forward Genetic Screening Identifies a Small Molecule That Blocks \textit{Toxoplasma gondii} Growth by Inhibiting Both Host- and Parasite-Encoded Kinases

Kevin M. Brown\textsuperscript{1,}\textsuperscript{*}, Elena Suvorova\textsuperscript{2}, Andrew Farrell\textsuperscript{3}, Aaron McLain\textsuperscript{4}, Ashley Dittmar\textsuperscript{4}, Graham B. Wiley\textsuperscript{5}, Gabor Marth\textsuperscript{3}, Patrick M. Gaffney\textsuperscript{5}, Marc Jan Gubbels\textsuperscript{3}, Michael White\textsuperscript{2}, Ira J. Blader\textsuperscript{1,4,}\textsuperscript{*}

\textsuperscript{1}Department of Microbiology and Immunology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma, United States of America, \textsuperscript{2}Departments of Molecular Medicine & Global Health, University of South Florida, Tampa, Florida, United States of America, \textsuperscript{3}Department of Biology, Boston College, Chestnut Hill, Massachusetts, United States of America, \textsuperscript{4}Department of Microbiology and Immunology, University at Buffalo, Buffalo, New York, United States of America, \textsuperscript{5}Arthritis and Clinical Immunology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma, United States of America

Abstract

The simultaneous targeting of host and pathogen processes represents an untapped approach for the treatment of intracellular infections. Hypoxia-inducible factor-1 (HIF-1) is a host cell transcription factor that is activated by and required for the growth of the intracellular protozoan parasite \textit{Toxoplasma gondii} at physiological oxygen levels. Parasite activation of HIF-1 is blocked by inhibiting the family of closely related Activin-Like Kinase (ALK) host cell receptors ALK4, ALK5, and ALK7, which was determined in part by use of an ALK4,5,7 inhibitor named SB505124. Besides inhibiting HIF-1 activation, SB505124 also potently blocks parasite replication under normoxic conditions. To determine whether SB505124 inhibition of parasite growth was exclusively due to inhibition of ALK4,5,7 or because the drug inhibited a second kinase, SB505124-resistant parasites were isolated by chemical mutagenesis. Whole-genome sequencing of these mutants revealed mutations in the \textit{Toxoplasma} MAP kinase, TgMAPK1. Allelic replacement of mutant TgMAPK1 alleles into wild-type parasites was sufficient to confer SB505124 resistance. SB505124 independently impacts TgMAPK1 and ALK4,5,7 signaling since drug resistant parasites could not activate HIF-1 in the presence of SB505124 or grow in HIF-1 deficient cells. In addition, TgMAPK1 kinase activity is inhibited by SB505124. Finally, mice treated with SB505124 had significantly lower tissue burdens following \textit{Toxoplasma} infection. These data therefore identify SB505124 as a novel small molecule inhibitor that acts by targeting two distinct targets, host HIF-1 and TgMAPK1.

For tachyzoites to be able to grow, they must establish a replicative niche within their host cells and do so by inducing a number of changes to host cell signaling, transcription, and organellar/cytoskeletal organization [9–17]. One of these changes includes activation of the host cell transcription factor hypoxia-inducible factor 1 (HIF-1), which is important for parasite growth [18]. \textit{Toxoplasma} activates HIF-1 by stabilizing the abundance of the HIF-1α subunit. HIF-1α stabilization is accomplished by the parasite down regulating the abundance and activity of the Proyl Hydroxylase Domain 2 (PHD2) protein whose primary function is to target HIF-1α for proteosomal-dependent degradation [19,20]. To down regulate PHD2, \textit{Toxoplasma} requires signaling via a family of host serine/threonine kinase receptors named the activin-like kinases (ALK4, ALK5, and ALK7) [21].

SB505124 [2-(5-benzo[1,5]dioxol-5-yl-2-tert-butyl-3H-imidazol-4-yl)-6-methylpyridine hydrochloride], which is a highly selective ALK4,5,7 competitive inhibitor, was one reagent used to demonstrate a role for ALK4,5,7 signaling in HIF-1 activation [21,22]. But besides inhibiting ALK4,5,7/HIF-1, SB505124 also

Introduction

\textit{Toxoplasma gondii} infects approximately one-third of the world's population and causes disease primarily in developing fetuses or immunocompromised individuals [1]. Humans and other intermediate hosts are infected with \textit{Toxoplasma} by digesting either sporozoite-containing oocysts that are shed in feline fecal matter or bradyzoite-laden tissue cysts in undercooked meat [2]. In the intestine, the parasites infect intestinal epithelial cells and then convert into the replicative form of the parasite called tachyzoites [3]. As tachyzoites disseminate through the host, they encounter various host defenses or pharmacological agents that in most cases kill the tachyzoites. However, some escape killing and transform into bradyzoites that go on to develop into tissue cysts. These tissue cysts cannot be detected by the host's immune system and are impervious to most, if not all, currently prescribed drugs [3–8]. Thus, \textit{Toxoplasma} is highly prevalent in humans, in large part, because tachyzoites have evolved to grow within its host until it is challenged and then respond by forming a life-long chronic infection.
Understanding how a compound blocks growth of an intracellular pathogen is important not only for developing these compounds into drugs that can be prescribed to patients, but also because these data will likely provide novel insight into the biology of these pathogens. Forward genetic screens are one established approach towards defining these mechanisms. But performing these screens with intracellular parasites has been limited not only because of technical limitations but also because the compounds may have off-target effects in either the host or parasite. Here, we report the first compound that kills a pathogen by simultaneously inhibiting distinct host- and parasite-encoded targets. Because developing drug resistance simultaneously to two targets is less likely, this work may highlight a new approach to antimicrobial drug discovery.

To define how SB505124 affected parasite growth, we developed a forward genetic screen to isolate and characterize SB505124 resistant parasites. This screen revealed that SB505124 inhibits *Toxoplasma* growth by not only inhibiting ALK4,5,7/HIF-1 signaling but by targeting a parasite MAP kinase named TgMAPK1.

**Results**

**Isolation of SB505124-Resistant Parasites**

To define how SB505124 inhibited parasite growth, we developed a forward genetic screen to isolate SB505124-resistant (SBR) parasites. Wild-type RH AX HX GPRT (RH) parasites were chemically mutagenized with N-Ethyl-N-nitrosourea (ENU) and SBR mutants were isolated by growth in the presence of SB505124. Three SBR mutants (SBR1, SBR2, and SBR3) were isolated from three independent mutagenesis screens and relative resistance of each mutant to SB505124 was determined by plaqueing assays. SBR mutants displayed similar IC_{50} values ranging from 4.9 μM to 5.4 μM, which relative to the parental RH strain represented 5-fold increases in resistance to SB505124 (Figure 1A). Unless stated otherwise, the remaining assays in this report were performed with SBR1.

To define how SB505124 affected parasite growth, mock- or drug-treated parasites were stained with DAPI and anti-SAG1 antisera to assess DNA content and identify individual parasites by immunofluorescence. SAG1 staining revealed that, as we previously reported [21], 3 μM SB505124 caused parasites to arrest growth as single SAG1+ parasites (Figures 1B&C). However, DAPI staining indicated that most vacuoles, including the seemingly single SAG1+ parasites, contained irregular numbers (non-2n) of parasites/vacuole (Figure 1C “Irregular Vacuole”). Numbers of nuclei/parasite bodies of those parasites growing within the “Irregular Vacuoles” were quantified (Figure 1D). Approximately 65% of vacuoles from the drug-treated RHΔ parasites contained ≥2 nuclei/parasite body. We also noted that approximately 25% of the SB505124-treated vacuoles contained parasites that were SAG1+/DAPI−, which are denoted as <1 nucleus/parasite body. In contrast, SBR1 replication was neither affected by SB505124 nor did the drug trigger an accumulation of multinucleated parasites or irregular vacuoles (Figure 1C&D). These data suggest that SB505124 disrupts tachyzoite cell cycle progression.

SB505124 is a substituted imidazole compound that was developed from the same structural scaffold as the p38 MAP kinase inhibitor SB203580 [22]. SB203580 inhibits *Toxoplasma* replication and does so presumably by targeting a SB203580-sensitive, parasite-encoded kinase [23,24]. Even though SB505124 more potently inhibits ALK4,5,7 than human p38 MAP kinases [22], it was possible that SB505124 reduced parasite growth by targeting the same kinase that SB203580 inhibits. A plaquing growth assay, however, revealed that SB203580 similarly inhibited RHA and SBR1 growth (IC_{50}~15 μM) (Supplemental Figure S1), which is consistent with earlier reports [23]. Thus, these two structurally related kinase inhibitors appear to inhibit *Toxoplasma* through distinct mechanisms and target proteins.

The preceding assays were performed with RH strain parasites because it is the strain best suited for genetic manipulation. But one limitation of the RH strain is that it grows exclusively as tachyzoites and does not readily undergo bradyzoite differentiation either in vitro or in vivo as other parasite strains can. Before testing the effect of SB505124 on the ability of the other parasite strains to undergo bradyzoite development, we first assessed whether SB505124 inhibited their proliferation as tachyzoites. We therefore grew Pru (a Type II strain), CTG (a Type III strain), and GT-1 (a Type I strain) in the presence of 3 μM SB505124 and determined the drug’s IC_{50} towards each strain (Figure 2A). SB505124 blocked lytic replication of all 3 strains with IC_{50} values lower than RH’s IC_{50}.

To assess the impact of SB505124 on bradyzoite development, GT-1, Pru, and CTG tachyzoites were grown on coverslips in the presence of SB505124. After 72 hours, parasites were fixed and stained with Dolichos-FITC to detect bradyzoite-containing cysts. We found that 2 μM SB505124 induced the formation of Dolichos’ cysts (Figure 2B and Supplemental Figure S2). But unlike high pH-induced bradyzoites, SAG1 staining could still be detected in the encysted parasites. To further assess bradyzoite development, Pru parasites were grown for 72 h in human foreskin fibroblasts (HFFs) in the presence of 3 μM SB505124 and bradyzoite specific gene expression was assessed by real time PCR. The data indicated that SB505124 induced a several hundred-fold increase of the bradyzoite gene transcripts, BAG1, LDH2, and ENO1 (Figure 2C). While a down-regulated trend was observed for the bradyzoite-specific gene ENO2, decreases in its abundance were not statistically significant (Figure 2C). Thus, SB505124 treatment induces some, but not all, features of bradyzoite development in cystogenic strains of *Toxoplasma*.

**TgMAPK1 Is a SBR Gene**

To identify the genetic basis for SB505124 resistance, genomic DNA purified from each SBR mutant and the parental RH parasites were subjected to Illumina whole genome sequencing. The sequenced genomes were aligned to the GT-1 *Toxoplasma gondii* reference genome and compared to identify ENU-induced single nucleotide variants (insertions or deletions were not detected) (Supplemental Table S1). To prioritize testing of candidate SBR mutations, we searched for genes that were mutated in more than one SBR mutant. From this filter, only one gene, *Toxoplasma* MAP Kinase 1 (TgMAPK1; TGGT1_312570 @ www.toxodb.org), was mutated in all three SBR mutants.
Figure 1. Generation of SBR mutants. A. Relative plaque formation in HFFs was determined for each parasite strain in the presence of increasing concentrations of SB505124. B–D. Parasite replication was measured by infecting HFFs on glass coverslips in the presence or absence of 3 μM SB505124 and then fixing the cells 24 hours later. Parasites and nuclei were detected with anti-SAG1 antibody and DAPI, respectively. B. Representative images. C. For each replicate, 100 vacuoles were monitored for parasites per vacuole and nuclei per parasite. Vacuoles were designated as being irregular if they contained an irregular number of parasites/vacuole (non 2^n). Shown are averaged percentages and standard deviations of 2 independent experiments with two replicates each. D. Averaged percentages and standard deviations of irregular vacuoles (show in C) by nuclei per parasite.
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Figure 2. SB505124 induces bradyzoite development. A. HFFs growing in 24 well plates were infected with RH, GT1, Pru, and CTG. After 5–7 days, monolayers were methanol-fixed, stained with crystal violet, and plaques were counted. Means and standard deviations are shown. B. HFFs grown on glass coverslips were infected with Pru strain parasites and either mock-treated or treated with pH 8.2 media or 3 μM SB505124. After 72 h, cells were fixed and stained with anti-SAG1 antisera and Dolichos-FITC to visualize cysts. Shown are representative images. C. HFFs were infected with Pru and either mock-treated or treated with pH 8.2 media or 3 μM SB505124. After 72 h, parasites were released from host cells, washed, and lysed. Total RNA was extracted, DNase I-treated and converted to cDNA. Relative amounts of transcripts were determined by Real Time PCR using β-actin as an internal reference. Fold change for each gene’s transcript abundance is reported as $2^{-\Delta\Delta Ct}$. Shown are averaged data with standard deviations from 3 experiments. Note that changes in ENO2 are not statistically significant.

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(Figures 3A&B and Supplemental Table S1). These three mutations affected the translation of two amino acids (Leu162→Gln and Ile171→Thr or Asn) in exon 2 that are part of the kinase’s predicted ATP binding pocket within its catalytic domain.

To determine whether these TgMAPK1 mutations were bona fide SBR alleles, we used an allelic replacement strategy to test whether replacing the wild-type TgMAPK1 allele with the mutant one conferred SB505124 resistance (Figure 3C). Thus, RHΔku80 parasites were transfected with TgMAPK1WT or TgMAPK1SBR allelic replacement constructs consisting of 900 bp fragments of the TgMAPK1 gene containing either WT or candidate SBR alleles. The transfected parasites were grown in the presence of 3 μM SB505124 and parasite growth was monitored visually.

**Figure 3. TgMAPK1 is an SBR gene.** A. Venn diagram of whole genome sequencing data of codon-changing SNVs identified in each mutant. B. Amino acid positions of TgMAPK1SBR mutations. C. TgMAPK1SBR allelic replacement strategy. Primers 1 and 2 were used to amplify 944 bp fragments of genomic DNA containing the SBR allele and cloned into pCR2.1. Primers 3 and 4 were used to amplify 1055 bp fragments of genomic DNA to confirm allelic replacement by Sanger sequencing. D. RHΔku80 parasites were transfected with linearized TgMAPK1WT or TgMAPK1SBR replacement constructs and grown in 3 μM SB505124-treated HFFs. Shown are representative images depicting the ability of RHΔku80:TgMAPK1SBR1 to grow and form plaques after 5 days of growth in the presence of 3 μM SB505124.

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SB505124 inhibited TgMAPK1 autokinase activity with an apparent IC_{50} of 125 nM (Figure 5D&E).

To address the possibility that the TgMAPK1 immunoprecipitates contained a contaminating kinase whose activity was SB505124 sensitive, we attempted to ectopically express a kinase-dead TgMAPK1 mutant cDNA by mutating the conserved lyse\textsuperscript{140} in the catalytic domain to an arginine. Repeated attempts to either transiently or stably express this mutant were unsuccessful as were attempts to express SBR mutants that were epitope tagged as either transgenics or at the endogenous TgMAPK1 locus. We therefore turned our attention to a TgMAPK1 temperature sensitive mutant that was isolated independently of this study in a temperature sensitive growth screen (LE and MW; manuscript in preparation). To minimize potential misfolding of the TgMAPK1\textsuperscript{ts} protein, autokinase activity was compared between parasites grown at 34°C (the permissive growth temperature for the ts mutant) that harbored either HA-tagged TgMAPK1\textsuperscript{wt} or TgMAPK1\textsuperscript{ts} alleles. Unlike TgMAPK1 kinase-dead and SBR mutants, TgMAPK1\textsuperscript{ts-HA} could be stably expressed and similar amounts of TgMAPK1\textsuperscript{WT-HA} or TgMAPK1\textsuperscript{ts-HA} could be immunoprecipitated using anti-HA beads (Figure 5F). In contrast, significantly lower levels of autokinase activity could be detected in the TgMAPK1\textsuperscript{ts} immunoprecipitates suggesting that the kinase activity in the immunoprecipitates was most likely dependent on TgMAPK1 and not a contaminating kinase (Figure 5G). Even though the wild-type and ts mutants grew at similar rates at 34°C we cannot fully rule out the possibility that potential misfolding of TgMAPK1\textsuperscript{ts} at the permissive temperature may affecting binding of a contaminating kinase. The differences in autokinase activity between the wild-type and ts kinases and the fact that the SBR mutations are in the region of the ATP binding pocket where the SB class of kinase inhibitors interact with their target kinases [27–29] strongly suggests that TgMAPK1 is an active kinase that can be inhibited by SB505124.

SB505124 Reduces Parasite Tissue Burden

Our in vitro data suggests that SB505124 blocks Toxoplasma growth in an unusual manner by which both host and parasite pathways are simultaneously targeted. To assess whether SB505124 can limit parasite growth in vivo, C57Bl/6 mice were intraperitoneally infected with 10^7 GFP-expressing RH tachyzoites and then treated with daily intraperitoneal injections of either 10 mg/kg SB505124 or vehicle (DMSO). On Day 5 post-infection, mice were sacrificed and peritoneal exudate cells were stained with anti-CD45 (to identify infiltrating leukocytes) and analyzed by flow cytometry. Mice treated with SB505124 had significantly fewer numbers of infected cells (12.8%) compared to the vehicle controls (25.7%) as measured by GFP/CD45^+ events (p = 0.0252) (Figures 6A,B). To determine whether the infected cells contained similar numbers of parasites, we measured the mean fluorescence intensity (MFI) of GFP in CD45^+ cells and found that infected cells from SB505124-treated mice displayed a 26.8% reduction in relative GFP MFI compared to vehicle-treated control mice (p = 0.0989) (Figure 6C). Though this decrease was not statistically significant, it is likely an underestimation as SB505124-treated parasites display enlarged, translationally active parasite bodies that could still produce high levels of GFP. The polyploidy phenotype induced by SB505124 precluded us from further assessing parasite burdens by qPCR analysis of genomic DNA.

Since SB505124 inhibits TGF\beta signaling, a known antagonist of IFN\gamma production [22,30], it is possible that the effect of SB505124 on Toxoplasma in mice is due to enhanced IFN\gamma-driven immunity. However, IFN\gamma serum levels from the mice 5 days post infection...
were identical between SB505124 and vehicle-control treated mice (Figure 6D).

**Discussion**

Our initial interest in SB505124 stemmed from finding that this compound blocked the parasite’s ability to activate HIF-1 via ALK4,5,7 signaling [21]. This conclusion was supported by additional data showing that overexpression of SMAD7, which is an endogenous ALK4,5,7 inhibitor, also blocked parasite activation of HIF-1 [21]. Additional unpublished work demonstrated that HIF-1 activity is increased in cells transfected with ALK4,5,7 expression plasmids. But since parasite replication was more severely reduced in SB505124-treated cells than in HIF-1α knockout cells, these data suggested that in addition to the ALK4,5,7/HIF-1 pathway the drug had at least one additional target that could be in either the host or parasite. Our forward genetic screen resolved this issue and identified SB505124 as the first compound that we are aware of that inhibits growth of an intracellular pathogen by acting on seemingly unrelated targets in both the host and parasite. We believe that collectively our data demonstrate that TgMAPK1 and ALK4,5,7/HIF-1 are the two

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**Figure 4. SB505124 impacts HIF1 and TgMAPK1 through distinct pathways.** A. HIF-1 luciferase reporter activity in mock- or SB505124-treated MEFs was measured after 18 h of infection with RHΔ or SBR1-3. Shown are averaged measurements and standard deviations from at least 3 independent experiments performed in triplicate. B. HIF-1α WT and HIF-1α -/- MEFs were grown in 24 well plates and infected with RHΔ or SBR1-3. The plates were grown for 66 h at 3% O₂ and then 5 μCi ³H-Uracil was added to each well to assess parasite growth. Shown are averaged data and standard deviations from 3 independent experiments performed in duplicate. C. HFFs grown on glass coverslips were infected with RH GFP at an MOI of 10 for 6 h in the presence or absence of 5 μM SB505124. Representative images are shown.

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Figure 5. SB505124 directly targets TgMAPK1. A. Strategy for endogenously tagging TgMAPK1 with 3×HA tag. B. Immunoprecipitated TgMAPK1-HA was separated by SDS-PAGE for western blotting antibody and in vitro kinase assays. C. Equivalent volumes of RH WT or RH:TgMAPK1-HA lysate were added anti-HA sepharose beads, then washed and then processed for in vitro autokinase assays. The lysates were then separated by SDS-PAGE and visualized by autoradiography. Shown is a representative assay. D. Equivalent amounts of TgMAPK1-HA was immunoprecipitated from RH:TgMAPK1-HA lysates using anti-HA sepharose beads and processed for in vitro kinase assays in the presence of increasing concentrations of SB505124. A representative assay with relative amounts of TgMAPK1 activity in each reaction is shown. E. Dose response curve showing averaged data and standard deviations from 3 experiments. F. Lysates (80 μg) were prepared from RH ΔKu80ΔHPT (WT), RH ΔKu80ΔHPT:TgMAPK1WT-HA, and RH ΔKu80ΔHPT:TgMAPK1TS-HA parasites grown at 34°C. Epitope-tagged TgMAPK1 was then detected using rat anti-HA antisera in the whole cell lysate and the flow through and immunoprecipitates following immunoprecipitation using rabbit anti-HA antibody conjugated beads. G. Immunoprecipitates of the indicated HA-tagged TgMAPK1 alleles were washed in kinase assay buffer and then incubated with γ32P-ATP for 60′ at 34°C. Shown are triplicate samples prepared from the same lysates immunoprecipitated in F. The experiment was repeated 3 independent times and representative gels are shown.

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relevant SB505124 targets in parasite-infected cells. While we cannot rule out that the drug may have additional targets in *Toxoplasma*-infected cells, these targets would not be important for parasite growth.

Although the catalytic domain of TgMAPK1 is homologous to other MAPKs, it has two unusual features that may facilitate TgMAPK1-specific agonist development. First, TgMAPK1 has 3 unique insertions within its catalytic domain with the most predominant one being a 93 amino acid insertion between the DFGLAR-motif that coordinates divalent cations. How the catalytic domain of the kinase can properly fold with this large insertion is currently unknown and cannot be easily predicted since the insertion prevents protein-modeling programs from using known MAPK structures as scaffolds (Brown and Blader, unpublished results). Second, TgMAPK1 has an 800 amino acid C-terminal extension that lacks conserved domains and has no homology to any known protein either in *Toxoplasma* or its host. Given that inhibition of TgMAPK1 induces multinucleated

**Figure 6. SB505124 reduces parasite growth in *Toxoplasma*-infected mice.** RH-GFP infected mice were intraperitoneally injected daily with 10 mg/kg SB505124 or DMSO alone. After 5 days post-infection, mice were sacrificed and flow cytometric analysis was performed on peritoneal exudate cells (3–4 mice per treatment group per experiment, 2 independent experiments). A. FACS plots (upper) and histograms (lower) showing percentages of infected (GFP+) cells of two representative mice per treatment group. B. Mean percentages of infected cells between treatment groups with standard deviations. C. Relative MFI of infected (GFP+) cells with standard deviations. D. ELISA determination of serum IFNγ levels of mock- and drug-treated, intraperitoneally infected mice 5 days post-infection. Shown are average and standard deviations.

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parasites (suggesting a cell cycle defect) and that the C-terminal extension facilitates its localization to mitotic structures (E. Suvorovo et al, manuscript in preparation) we hypothesize that TgMAPK1 functions in cell cycle regulation and our future work will define if and how TgMAPK1 regulates the cell cycle.

TgMAPK1 was originally discovered based on its homology to human MAPKs [24]. It was assigned then as a p38 MAPK homolog, in part, because its catalytic activity was reported to be sensitive to SB203580. In our hands, TgMAPK1 activity was largely unaffected by this compound (Supplemental Figure S1B), although a general decrease in autokinase activity by low SB203580 levels was noted that may reflect reduced non-specific binding of ATP to the kinase. Regardless, higher amounts of SB203580 did not significantly decrease TgMAPK1 autokinase activity and SBR mutants are similarly sensitive to SB203580 (Supplemental Figure S1A&B). We believe that there are two primary reasons for differences in between our data and those of [24]. First, our experiments used endogenously epitope-tagged TgMAPK1 that was immunoprecipitated from tachyzoites. In contrast, Brumlik and colleagues used a bacterially expressed construct that only contained TgMAPK1’s catalytic domain and most of this protein was expressed in bacterial inclusion bodies (our unpublished observations) [24]. Even though we could purify the protein from the inclusion bodies under denaturing conditions, very weak kinase activity was detected after refolding and we could not verify whether this protein was properly folded. Attempts to purify the soluble protein resulted in a preparation of poor purity (as noted by [24]) whose activity appeared to be due to a contaminating bacterial kinase since recombinant expression of a catalytically inactive kinase mutant had the same rate of activity as the wild-type kinase (not shown). Recently, Sugi et al demonstrated that the SBR2 (Leu162→Gln) and SBR3 (Ile171→Asn) alleles conferred resistance to the effect of the bumped kinase inhibitor 1NM-PP1 on parasite replication [25]. TgMAPK1 resistance to 1NM-PP1 is independent of its well-described inhibition of T. gondii Calcium-Dependent Protein Kinase 1, whose activity is required for parasite invasion and egress [31]. But unlike our work, Sugi et al. did not test whether 1NM-PP1 affects parasite replication by directly inhibiting TgMAPK1 or if resistance emerged because TgMAPK1 functions downstream of the protein(s) that 1NM-PP1 interacts with [25,32]. It is also noteworthy that we were unable to successfully express a kinase-dead mutant of TgMAPK1 as a transgene either in transiently or stably transfected parasites. The most likely explanation for this is that expression of the kinase dead TgMAPK1 mutant has a dominant negative effect either by binding its upstream activating kinase or its downstream targets.

The continuing emergence of antimicrobial resistance requires novel approaches to the design of new drugs and treatments. Although simultaneous inhibition of both host and parasite targets is an untested approach, one benefit would be that resistance to a compound that impacts a host cell target is less likely to develop in parasites from activating ALK4,5,7 or prevent these receptors from triggering HIF-1 activity. We believe that either of these approaches would be valid even if SB505124 induces bradyzoite development as a consequence of the drug activating a more generalized stress response. Our future analysis of TgMAPK1 regulation and substrate identification will resolve how TgMAPK1 influences bradyzoite differentiation.

In summary, we used a forward genetic screen to demonstrate that a serine/threonine kinase inhibitor blocks T. gondii growth through two distinct targets and our future work will focus on this issue. Kinase inhibitors are particularly useful for dual-target screens because even though they are designed to inhibit human kinases their off target effects cannot be predicted [37]. For example, we showed that SB203580 does not inhibit TgMAPK1, which bears homology to p38 MAPKs. Rather TgMAPK1 is inhibited by SB505124 even though this compound does not appear to significantly affect p38 MAPK activity [22]. Our work, therefore, also highlights the utility of repurposing drugs and investigative compounds originally developed to target cancer and other diseases for the study and/or treatment of microbial infections [38–41].

Materials and Methods

Ethics

Animal protocols (IACUC Protocol #11-075J) were approved by the University of Oklahoma Health Sciences Center IACUC. This study was carried out in strict accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and AAALAC accreditation guidelines.

Cell and Parasites

Toxoplasma RH, RHAHXGPR T, RHA-GFP (kindly provided by Dr. Gustavo Arrizabalaga of Indiana University), RHA RHAIHXGPR TAku80 (kindly provided by Dr. David Bzik of Dartmouth University), GT-1, Pru, and CTG parasites were maintained in human foreskin fibroblasts in Dulbecco’s Modification of Eagle’s Medium (DMEM) (Mediatech; Manassas, VA) supplemented with 10% fetal bovine serum (Mediatech), 2 mM L-glutamine (Mediatech), and 100 IU/ml penicillin – 100 μg/ml streptomycin (Mediatech). The TgMAPK1ts-HA temperature sensitive (Δ2) TgMAPK1 mutant will be described in detail...
elsewhere (manuscript in preparation). Other host cell lines were maintained in the HFF growth medium. All host cell lines and parasites were routinely tested for Mycoplasma contamination with the MycoAlert Mycoplasma Detection Kit (Lonza, Basel, Switzerland) and found to be negative.

Chemical Mutagenesis

Intracellular RH69XGPRT tachyzoites grown in T-75 flasks were mutagenized with 300 μg/ml ENU (Sigma; St. Louis, MO) in complete DMEM for 2 hours at 37°C as previously described [42,43]. After ENU treatment, media was removed and the monolayer was washed four times with 1× phosphate-buffered saline. Mutagenized parasites were then released from host cells by scraping and syringe-lysis (27 g needle) and washed with 30 ml DMEM. Parasites were allowed to recover in fresh HFFs for 72 h and then grown in the presence of SB505124 (TGF-βRI Kinase Inhibitor III [EMD Millipore; Billerica, MA]). After several rounds of selection in SB505124, individual clones were obtained by limiting dilution in 96 well plates of HFFs. SBR1 was isolated from a population after 4 passages in 3 μM SB505124 and 11 passages in 5 μM SB505124. SBR2 was isolated after 4 passages in 5 μM SB505124. SBR3 was isolated after 5 passages in 5 μM SB505124.

Growth Assays

Plate assays were performed by adding 200 parasites to each well of a 24 well plate containing confluent HFFs. After 5–7 days, the monolayers were methanol-fixed and stained with 0.1% crystal violet. Plaques were counted using 4× magnification on an inverted dissecting microscope. Uracil incorporation assays were performed by growing parasites in 24 well plates. After 66 h, 5 μCi 3H-uracil (MP Biomedical; Santa Ana, CA) was added to the wells for an additional 6 h of the assay. Wells were washed, precipitated with 10% trichloroacetic acid, and 3H-uracil counted by liquid scintillation.

Whole Genome Sequencing

Parasites were grown in confluent HFFs until the host monolayer had completely lysed. The media containing the extracellular parasites were collected without further scraping, washed with serum-free DMEM, passed three times through a 27 g needle, and then filtered through a 3 μm pore size filter membrane. Genomic DNA was isolated using the Qiamp DNA mini kit (Qiagen; Valencia, CA) using the manufacturer’s protocol for cultured cells. qRT-PCR (not shown) determined that host DNA contamination was less than 0.5% and this result was confirmed by the whole genome sequencing data. Sequencing libraries were prepared from genomic DNA (1 μg) using the TrueSeq DNA LT Sample Prep Kit v2 as per the manufacturer’s protocols (Illumina, San Diego, CA) and the libraries sequenced on an Illumina HiSeq 2000 instrument with 100 bp, paired-end reads. Between 30–37-fold coverage was achieved for all genomes. Genome sequences were aligned with the Mosaik program [44,45] using the type I parasite genome of GT-1 as a reference and single nucleotide variants identified with variant caller program FreeBayes [46]. For Mosaik, the default parameters we used had a hash size of 14. For FreeBayes, we used the program’s default parameters with the exception that: ploidy (-p) was set to 1, a P value cutoff of 0.9 (-P) was used, complex events were ignored (-a), and population priors were turned off (-no-population-priors) as they are not applicable to the Toxoplasma genome.

Allelic Replacement of TgMAPK1SBR Mutations

A 944 bp fragment of TgMAPK1 was PCR amplified using Platinum Pf polymerase (Invitrogen; Carlsbad, CA) from wild-type and SBR mutant parasites with forward (5’TGCATGGCGATGAGTTTCTGAACG-3’) and reverse (5’TCTGTTGTCAGCCTTTCGTGGA-3’) primers. PCR reactions were incubated with Taq polymerase (Invitrogen) for 10 s, followed by 3’ A-overhangs. PCR products were gel purified and cloned into pCR2.1 (Invitrogen) by TA ligation. Inserts were sequenced verified by conventional sequencing.

Parasites were transfected with 50 μg of linearized plasmid resuspended in cytomix buffer (2 mM ATP, 5 mM glutathione). For each transfection, 2×107 RH69XGPRTAKa10 tachyzoites were washed in cytomix buffer and resuspended in 0.5 ml complete cytomix buffer. The plasmid DNA was added to the parasites in a BTX electroporation cuvette (0.4 mm gap), and electroporated into the parasites to 2000 V, 50 ohm, 25 μF with a BTX ECM 360 (Holliston, MA). Parasites were then transferred to 75 cm2 flasks containing confluent HFF monolayers and after 72 h 3 μM SB505124 was added. Parasites receiving pCR2.1:TgMAPK1WT failed to grow whereas those that received pCR2.1:TgMAPK1SBR1-3 were able to be passaged indefinitely in 3 μM SB505124. Individual clones were isolated by limiting dilution as described above and single clones were named RH:MAPK1SBR1, RH:MAPK1SBR2, and RH:MAPK1SBR3. To ensure that the SBR mutation was incorporated into the endogenous MAPK1 locus, PCR fragments were generated from genomic DNA using primers that flanked the region of the amplicon. The amplicon was then gel purified and cloned into pCR2.1. At least 5 independent transformants were sequenced via conventional Sanger sequencing using M13 forward and reverse universal primers.

Fluorescence Microscopy

RH clones were added to 24 well plates containing confluent HFFs on glass coverslips in the presence or absence of 3 μM SB505124. After 24 h, coverslips were methanol-fixed, blocked with 3% bovine serum albumin, and labeled with rabbit anti-SAG1 (1:10000; kindly provided by Dr. John Boothroyd, Stanford University) followed by Alexa Fluor 488-conjugated goat anti-rabbit (Invitrogen). Coverslips were mounted to slides with Vectashield containing DAPI (Vector Lab; Burlingame, CA). One hundred randomly chosen vacuoles/overlap were counted.

Phospho-STAT3 was detected in HFFs infected with RH-GFP (MOI of 10) tachyzoites for 6 h in the absence or presence of 5 μM SB505124. Cells were fixed with 4% formaldehyde, permeabilized with ice-cold methanol for 5 minutes, blocked at room temperature for 2 h with 3% BSA, and incubated with rabbit anti-phosphoSTAT3 (Cell Signaling; Danvers, MA) overnight at 4°C. After washing, cells were stained with Alexa Fluor 594 conjugated goat-anti-rabbit (Invitrogen) and mounted to slides with Vectashield containing DAPI.

Bradyzoite cysts were detected in HFFs 72 h after adding either 3 μM SB505124 or pH 8.1 media [47]. Monolayers were fixed with ice-cold methanol for 5 minutes, blocked at room temperature for 2 h with 3% BSA, and incubated with rabbit anti-phosphoSTAT3 (Cell Signaling; Danvers, MA) overnight at 4°C. After washing, cells were stained with Alexa Fluor 488 conjugated goat anti-rabbit (Invitrogen) and mounted to slides with Vectashield containing DAPI.

In Vitro Kinase Assays

To generate strains in which TgMAPK1 is epitope tagged at its C-terminus with three HA repeats (RHAHXGPRTAKa10:Tg-
MAPK1-3XHA), a 1513 bp fragment was amplified from the 3’ end of TgMAPK1 using primers TgMAPK1-3XHA and TgMAPK1-3XHAR and cloned into p3xHA-LIC-HXGPRT by ligation independent cloning to create pTgMAPK1-3XHA [40]. RHAHXGPRTAu80 parasites were transfected with Pck-linearized TgMAPK1 plasmids and transfectants selected with mycophenolic acid/xanthine. Proper integration and expression was by PCR (not shown), Western blotting (Figure 5B), and immunodetection (E. Suvorova et al., Manuscript in Preparation).

25 cm² flasks were sufficient to detect and assay the wild-type parasites and 24 h later the parasites were harvested and lysed. Extracellular parasites were washed and syringed released by scraping and syringe lysis. The parasites were washed and lysed on ice with 200 μl modified RIPA buffer (50 mM Tris pH 7.4, 1% NP-40; 0.1% SDS, 500 mM NaCl) supplemented with 1× EDTA-free Protease Inhibitor Cocktail (Roche; Indianapolis, IN) per 10⁷ parasites. Lysates were centrifuged at 20,000 x g for 15’ at 4°C to remove insoluble material and the supernatant was preclared with rabbit IgG-conjugated sepharose beads (Cell Signaling). Precleared lysates were added to 10 μl anti-HA sepharose beads (Cell Signaling) and incubated overnight at 4°C, washed 3 times in modified RIPA buffer, and 1 time in kinase assay buffer (20 mM HEPES pH 7.4, 25 mM glycercophosphate, 25 mM MgCl₂, 0.5 mM DTT, and 0.1 mM Na₃VO₄). Immune complexes were then evenly distributed between sample tubes (the equivalent of 200 μg of preclared lysate was used for each sample) and incubated with 10 μCi γ³²P-ATP (MP Biomedicals) in kinase assay buffer at 34°C for 1 h. Kinase reactions were stopped by adding 5 μl 6x sample buffer and boiling the samples for 5’. Reactions were separated by SDS-PAGE (10% acrylamide) and gels were fixed for 20’ in acetic acid:methanol:H₂O (1:5:4) and dried. The gels were exposed to film and analyzed using ImageJ software.

We attempted to express kinase-dead TgMAPK1 (K140R) and SBR mutants as a transgenes by synthesizing full length wild-type and mutant constructs in frame with C-terminal 3x HA-tags (Genescript; Piscataway, NJ). The cDNA constructs were cloned into pSACGAT by replacing the CAT gene with the TgMAPK1 gene [49]. The constructs were then transfected into RH strain parasites and 24 h later the parasites were harvested and transgenic TgMAPK1 expression assessed by Western blotting and activity by in vitro kinase reaction. We were only able to detect low levels of mutant TgMAPK1 protein even when 175 cm² flasks were used for the transfection and this amount of kinase was not sufficient for the autokinase reactions (not shown). In contrast, 25 cm² flasks were sufficient to detect and assay the wild-type kinase. We next cloned the TgMAPK1 alleles into pTuHFYPYFP/sagCAT [48] by replacing the YFP genes with the kinase alleles. Using chloramphenicol selection, stable transforms could be isolated for those parasites receiving wild-type TgMAPK1 but we could not obtain transforms for parasites receiving mutant alleles.

We also attempted to epitope tag the SBR mutants at their endogenous locus by cloning the 1.2 kb 3’ most region (up to the stop codon) of the genomic region of the TgMAPK1 into pSF-TAP-LIC-HXG in frame with the SF-TAP tag [48]. This single construct is compatible with both the wild type and SBR strains since the 3’ region of homology is downstream of the SBR mutations. We linearized the vector with NcoI, a unique restriction site within the TgMAPK1 sequence giving 677 bp of homology to the endogenous TgMAPK1 allele, and transfected the DNA into RHAKu80AHXGPR/ TgMAPK11 without the parental wild type strain. But, we were unable to recover viable SBR parasites from selection with MPA/xanthine even though we were able to do so for the wild-type parasites.

### Western Blotting

Proteins were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with 5% BSA-TBST. Rat anti-HA (Roche) was used at a concentration of 1:5000. Goat anti-rat HRP (Cell Signaling) was used at a concentration of 1:2000 and detected using Supersignal West Pico ECL reagent (Thermo, Waltham, MA) with a gel doc system.

### Reverse Transcriptase Real Time-PCR

RNA was isolated with the RNeasy Midi kit (Qiagen) using the manufacturer’s instructions for cultured cells. RNA quality and yield was assessed by UV spectrophotometry and horizontal electrophoresis. Total RNA was treated with DNase I (Invitrogen) and converted to cDNA using Superscript III (Invitrogen) using random hexamer primers. Relative abundances of Bradyzoite gene transcripts BAG1, LDH2, ENO1 and tachyzoite transcript ENO2 were calculated with the 2⁻ΔΔCt method [50] using Toxoplasma β-actin as an internal control (see Supplemental Table S2 for primer sequences).

### HIF1 Reporter Assay

HIF-1 luciferase assays were performed as previously described [18]. Briefly, murine embryonic fibroblasts were transfected pHRE-luc and pTK-Rel (as a transfection efficiency control) and 24 h later 2×10⁶ parasites were added to the wells in the presence or absence of SB505124. 18 hours later, luciferase activity was measured using the Dual-Glo luciferase assay kit (Promega, Madison, WI).

### SB505124 In Vivo Experiments

C57Bl/6 female mice were infected intraperitoneally with 10⁵ freshly purified RH-GFP parasites in a volume of 200 μl phenol-red-free DMEM. Beginning 1 h post infection, mice received 10 mg/kg SB505124 or DMSO alone intraperitoneally once daily. The drug had no apparent effect on the health of the animals during this time course. On day 5 post-infection, mice were sacrificed by CO₂ asphyxiation and PECs collected in a 5 ml PBS-lavage. Cells were washed, stained with anti-mouse APC-conjugated CD45, and analyzed by flow cytometry using a FACSCalibur cytometer (BD Biosciences, San Jose, CA). 10⁵ events were counted and subsequent analysis was performed using Summit (Dako, Carpenteria, CA). IFNγ was measured using Mouse IFNγ ‘Femto-HS’ High Sensitivity ELISA (eBioscience; San Diego, CA).

### Supporting Information

**Figure S1** A. RHA or SBR1 parasites were grown in HFFs with the indicated concentrations of SB203580. After 5 days, the cells were fixed and numbers of plaques formed were determined by crystal violet staining. Shown are averages and standard deviations of 3 independent experiments performed in triplicate. B. Equivalent amounts of TgMAPK1-HA was immunoprecipitated from RH-TgMAPK1-HA lysates using anti-HA sepharose beads and processed for in vitro kinase assays in the presence of increasing concentrations of SB203580. (TIF)

**Figure S2** HFFs grown on glass coverslips were infected with GT-1 (A) or CTG (B) strain tachyzoites and then either mock-treated or treated with pH 8.2 media or 3 μM SB505124. After 72 h, cells were fixed and stained with anti-SAG1 and Dolichos-FITC to visualize cysts. Shown are representative images. C. Quantification of numbers of GT-1, Pru, and CTG strain parasites that were Dolichos (DBA) after exposure to SB505124. Note that images for Pru strain parasites are shown in Figure 2B. (TIFF)
Table S1 Summary of the whole genome sequencing of the parental and SBR mutants. The first sheet is a summary of the sequencing for each strain and the number of base pairs sequenced for parasite nuclear and mitochondrial genomes as well as the host genome. The other sheets list the position of each identified mutation, how the mutation affected the a change in protein sequence and, if applicable, the gene in which the mutation lies. (XLS)

Table S2 List of either PCR primers to clone TgMAPK1 constructs or of RT-PCR primers to assess bradyzoite gene expression. (DOCX)

References
1. Pereira-Chioccola VL, Vidal JE, Su C (2009) Toxoplasma gondii infection and cerebral toxoplamosis in HIV-infected patients. Future Microbiol 4: 1363–1379.
2. Hill D, Dubey JP (2002) Toxoplasma gondii: transmission, diagnosis and prevention. Clin Microbiol Infect 8: 634–640.
3. Weiss LM, Kim K (2000) The development and biology of bradyzoites of Toxoplasma gondii. Front Biosci 5: D391–405.
4. Bolme W, Herrmann J, Gross U (1994) Reduced replication of Toxoplasma gondii is necessary for induction of bradyzoite-specific antigens: a possible role for nitric oxide in triggering stage conversion. Infect Immun 62: 1761–1767.
5. Ferguson DJ, Hutchison WM (1987) The host-parasite relationship of Toxoplasma gondii in the brains of chronically infected mice. Virchows Arch A Pathol Anat Histopathol 411: 39–41.
6. Faucher B, Moreau J, Zareg F, Franck J, Parraux R (2011) Failure of conventional treatment with pyrimethamine and sulfadiazine for secondary prophylaxis of cerebral toxoplamosis in a patient with AIDS. J Antimicrob Chemother 66: 1653–1656.
7. Dunay IR, Heimseaaat MM, Bushbath FN, Muller RH, Stocker H, et al. (2004) Atovaquone maintenance therapy prevents reactivation of toxoplams ischemic in a murine model of reactivated toxoplamosis. Antimicrob Agents Chemother 48: 4859–4864.
8. Schaefiff M, Han SJ, Chmeirova T, van Dooren GG, Hertzmark P, et al. (2009) Dynamic imaging of T cell-parasite interactions in the brains of mice chronically infected with Toxoplasma gondii. J Immunol 182: 6379–6393.
9. Molestina RE, Payne TM, Coppens I, Sinai AP (2003) Activation of NF-kappaB by Toxoplasma gondii correlates with increased expression of antiaptopotic genes and localization of phosphorylated I-kappaB to the parastephophorous vacuole membrane. J Cell Sci 116: 4359–4371.
10. Del Rio L, Butcher BA, Bemmoua SA, Hnny Y, Saker A, et al. (2004) Toxoplasma gondii triggers myeloid differentiation factor 80-dependent NF-kappaB-12 and chemokine ligand 2 (monocyte chemotactic protein 1) responses using distinct parasite molecules and host receptors. J Immunol 170: 6954–6960.
11. Mason NJ, Fiore J, Kobayashi T, Masek KS, Chyi Y, et al. (2004) TRAF6-dependent mitogen-activated protein kinase activation differentially regulates the production of interleukin-12 by macrophages in response to Toxoplasma gondii. Infect Immun 72: 5662–5667.
12. Yamamoto M, Ma JS, Mueller C, Kamiyama N, Saiga H, et al. (2011) ATF6(betac) is a host cellular target of the Toxoplasma gondii virulence factor ROP18. J Exp Med 208: 1333–1346.
13. Yamamoto M, Standley DM, Takashima S, Saiga H, Okumaya M, et al. (2009) A simple polymeric amino acid on Toxoplasma gondii kinase ROP16 determines the direct and strain-specific activation of Stat3. J Exp Med 206: 2747–2760.
14. Blader IJ, Manger ID, Boothroyd JC (2001) Microarray Analysis Reveals Previously Unknown Changes in Toxoplasma gondii-infected Human Cells. J Biol Chem 276: 24223–24231.
15. Saeij JP, Czernecki K, Mazurek DS, Ehrmann CA, Roth MD, et al. (2007) Toxoplasma gondii sequesters lysosomes from mammalian hosts in the vacuolar space. J Cell Sci 116: 4359–4371.
16. Yamamoto M, Muller C, Kamiyama N, Saiga H, et al. (2011) ATFG6(beta) is a host cellular target of the Toxoplasma gondii virulence factor ROP16. J Exp Med 208: 1333–1346.
17. Blader IJ, Manger ID, Boothroyd JC (2001) Microarray Analysis Reveals Previously Unknown Changes in Toxoplasma gondii-infected Human Cells. J Biol Chem 276: 24223–24231.
18. Saeij JP, Coller S, Boyle JP, Jerome ME, White MW, et al. (2007) Toxoplasma co-opts host gene expression by injection of a polymorphic kinase homologue. Nature 445: 324–327.
19. Oppens I, Dunn JD, Romano JD, Pyaemt M, Zhang H, et al. (2006) Toxoplasma gondii sequesters lysosomes from mammalian hosts in the vacuolar space. Cell 125: 261–273.
20. Romano JD, Sonda S, Berghower E, Smith ME, Coppens I (2013) Toxoplasma gondii salvages sphingolipids from the host Golgi through the rerouting of cytoplasmic sphingolipids. Mol Biochem Parasitol 131: 119–127.
21. Saeij JP, Coller S, Boyle JP, Jerome ME, White MW, et al. (2007) Toxoplasma co-opts host gene expression by injection of a polymorphic kinase homologue. Nature 445: 324–327.
22. Oppens I, Dunn JD, Romano JD, Pyaemt M, Zhang H, et al. (2006) Toxoplasma gondii sequesters lysosomes from mammalian hosts in the vacuolar space. Cell 125: 261–273.
23. Berra E, Benizri E, Ginouves A, Volmat V, Roux D, et al. (2003) HIF-prolylhydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1alpha in normoxia. EMBO J 22: 4082–4090.
24. Appelhoff RJ, Tian YM, Raval RR, Turkey H, Harris AL, et al. (2004) Differential functional of the prolyl hydroxylases PHD1, PHD2, and PHD3 in the regulation of hypoxia-inducible factor. J Biol Chem 279: 38458–38465.
25. Schaeffer M, Han SJ, Chtanova T, van Dooren GG, Herzmark P, et al. (2009) Dynamic imaging of T cell-parasite interactions in the brains of mice chronically infected with Toxoplasma gondii. J Immunol 182: 6379–6393.
26. Del Rio L, Butcher BA, Bemmoua S, Hnny Y, Saker A, et al. (2004) Toxoplasma gondii triggers myeloid differentiation factor 80-dependent NF-kappaB-12 and chemokine ligand 2 (monocyte chemotactic protein 1) responses using distinct parasite molecules and host receptors. J Immunol 170: 6954–6960.
27. Mason NJ, Fiore J, Kobayashi T, Masek KS, Chyi Y, et al. (2004) TRAF6-dependent mitogen-activated protein kinase activation differentially regulates the production of interleukin-12 by macrophages in response to Toxoplasma gondii. Infect Immun 72: 5662–5667.
28. Yamamoto M, Ma JS, Mueller C, Kamiyama N, Saiga H, et al. (2011) ATFG6(betac) is a host cellular target of the Toxoplasma gondii virulence factor ROP18. J Exp Med 208: 1333–1346.
29. Yamamoto M, Standley DM, Takashima S, Saiga H, Okumaya M, et al. (2009) A simple polymeric amino acid on Toxoplasma gondii kinase ROP16 determines the direct and strain-specific activation of Stat3. J Exp Med 206: 2747–2760.
30. Blader IJ, Manger ID, Boothroyd JC (2001) Microarray Analysis Reveals Previously Unknown Changes in Toxoplasma gondii-infected Human Cells. J Biol Chem 276: 24223–24231.
31. Saeij JP, Coller S, Boyle JP, Jerome ME, White MW, et al. (2007) Toxoplasma co-opts host gene expression by injection of a polymorphic kinase homologue. Nature 445: 324–327.
32. Oppens I, Dunn JD, Romano JD, Pyaemt M, Zhang H, et al. (2006) Toxoplasma gondii sequesters lysosomes from mammalian hosts in the vacuolar space. Cell 125: 261–273.
33. Berra E, Benizri E, Ginouves A, Volmat V, Roux D, et al. (2003) HIF-prolylhydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1alpha in normoxia. EMBO J 22: 4082–4090.

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Author Contributions
Conceived and designed the experiments: KMB ES JB. Performed the experiments: KMB ES GBW PMG MJG MW JB. Analyzed the data: KMB ES GBW PMG MJG MW JB. Contributed reagents/materials/analysis tools: GM PMG MJG MW JB. Wrote the paper: KMB JB.
40. Debnath A, Parsonage D, Andrade RM, He C, Cobo ER, et al. (2012) A high-throughput drug screen for Entamoeba histolytica identifies a new lead and target. Nat Med 18: 956–960.

41. Patel G, Karver CE, Behera R, Guyett P, Sullenberger C, et al. (2013) Kinase scaffold repurposing for neglected disease drug discovery: Discovery of an efficacious, lapatanib-derived lead compound for trypanosomiasis. Journal of Medicinal Chemistry 56: 3820–3832.

42. Pfefferkorn ER, Pfefferkorn LC (1977) Toxoplasma gondii: characterization of a mutant resistant to 5-fluorodeoxyuridine. Exp Parasitol 42: 44–55.

43. Black MW, Arrizabalaga G, Boothroyd JC (2000) Ionophore-resistant mutants of Toxoplasma gondii reveal host cell permeabilization as an early event in egress. Mol Cell Biol 20: 9399–9408.

44. Smith DR, Quinlan AR, Peckham HE, Makowsky K, Tao W, et al. (2008) Rapid whole-genome mutational profiling using next-generation sequencing technologies. Genome Res 18: 1638–1642.

45. Hillier LW, Marth GT, Quinlan AR, Dooling D, Fewell G, et al. (2008) Whole-genome sequencing and variant discovery in C. elegans. Nat Methods 5: 183–188.

46. Garrison E, Marth GT (2012) Haplotype-based variant detection from short-read sequencing. ARXIV e-print arXiv:1207.3907. Available: http://arxiv.org/abs/1207.3907. Accessed 30 April 2014.

47. Soete M, Camus D, Dubremetz JF (1994) Experimental induction of bradyzoite-specific antigen expression and cyst formation by the RH strain of Toxoplasma gondii in vitro. Exp Parasitol 78: 361–370.

48. Huynh MH, Carruthers VR (2009) Tagging of endogenous genes in a Toxoplasma gondii strain lacking Ku80. Eukaryot Cell 8: 530–539.

49. Soldati D, Boothroyd JC (1993) Transient transfection and expression in the obligate intracellular parasite Toxoplasma gondii. Science 260: 349–352.

50. Phelps E, Sweeney K, Blader IJ (2008) Toxoplasma gondii Rhoptry Discharge Correlates with Activation of the EGR2 Host Cell Transcription Factor. Infection and Immunity 76: 4703–4712.