Novel Triazine JPC-2067-B Inhibits *Toxoplasma gondii* In Vitro and *In Vivo*

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**Abstract**

**Background and Methodology:** *Toxoplasma gondii* causes substantial morbidity, mortality, and costs for healthcare in the developed and developing world. Current medicines are not well tolerated and cause hypersensitivity reactions. The dihydrotiazine JPC-2067-B (4, 6-diamino-1, 2-dihydro-2, 2-dimethyl-1-(3’-2-chloro-, 4-trifluoromethoxyphenoxy)propyloxy)-1, 3, 5-triazine), which inhibits dihydrofolate reductase (DHFR), is highly effective against *Plasmodium falciparum*, *Plasmodium vivax*, and apicomplexans related to *T. gondii*. JPC-2067-B is the primary metabolite of the orally active biguanide JPC-2056 1-(3’-(2-chloro-4-trifluoromethoxyphenoxy)propyl oxy)-5-isopropylbiguanide, which is being advanced to clinical trials for malaria. Efficacy of the prodrug JPC-2056 and the active metabolite JPC-2067-B against *T. gondii* and *T. gondii* DHFR as well as toxicity toward mammalian cells were tested.

**Principal Findings and Conclusions:** Herein, we found that JPC-2067-B is highly effective against *T. gondii*. We demonstrate that JPC-2067-B inhibits *T. gondii* growth in culture (IC₅₀ 20 nM), inhibits the purified enzyme (IC₅₀ 6.5 nM), is more efficacious than pyrimethamine, and is cidal *in vitro*. JPC-2067-B administered parenterally and the orally administered prodrug (JPC-2056) are also effective against *T. gondii* tachyzoites *in vivo*. A molecular model of *T. gondii* DHFR-TS complexed with JPC-2067-B was developed. We found that the three main parasite clonal types and isolates from South and Central America, the United States, Canada, China, and Sri Lanka have the same amino acid sequences preserving key binding sites for the triazine.

**Significance:** JPC-2056/JPC-2067-B have potential to be more effective and possibly less toxic treatments for toxoplasmosis than currently available medicines.


Editor: Greg Matlashewski, McGill University, Canada

Received: February 6, 2007; Accepted: January 16, 2008; Published: March 5, 2008

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Funding: This work was supported by R01 AI43228, AI26912, and AI60360, The Research to Prevent Blindness Foundation, and gifts from the Kieweit, Blackmon, Brennan, Koshland, Langel, Morel, Rosenstein, Kapnick, Cussen, Lipskar, Taub, and Rooney-Alden families. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: DPJ and Jacobus Pharmaceutical Company are progressing JPC-2056 for the treatment of malaria. The other authors declare that they have no competing financial interests.

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Toxoplasmosis is a neglected tropical disease as well as a significant illness affecting persons throughout the world and new and improved medicines are greatly needed for this and other apicomplexan infections [1–40]. In developing tropical countries, the problems for persons with AIDS can be exacerbated due to lack of both anti-retroviral treatment and anti-*Toxoplasma gondii* treatment. In this setting, this opportunistic pathogen causes substantial neurologic disease and treatment of this illness can be especially difficult because current gold standard medicines are unobtainable and/or unaffordable and, due to their toxicity, require monitoring which exceeds the capacity of many of the available health care systems. Toxoplasmic eye disease (chorioretinitis) is frequent in certain areas of Brazil and Colombia, areas where the gold standard drugs are particularly problematic, and is caused by atypical parasites that present major recrudescent and recurrent clinical problems. *T. gondii* is highly pathogenic and lethal in an emerging problem in French Guiana and Suriname [22,34].

Throughout the world, new *T. gondii* infection during pregnancy can lead to devastating disease for the fetus and newborn infant, later impacting on the child’s health and development and potentially on his/her later productivity [1–3]. In all areas of the

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Author Summary

Toxoplasmosis is a neglected tropical disease, an emerging disease as well as a significant problem in developed countries causing a substantial health burden. Better medicines with less toxicity are greatly needed. Herein, we found that a novel triazine currently being advanced to clinical trials for malaria, JPC-2067-B, is highly effective against *T. gondii*. We demonstrate that JPC-2067-B inhibits *T. gondii* growth in culture (IC50 20 nM), inhibits the purified enzyme (IC50 6.5 nM), is more efficacious than pyrimethamine, and is cidal *in vitro*. JPC-2067-B administered parenterally and the orally administered pro-drug (JPC-2056) are also effective against *T. gondii* tachyzoites *in vivo*. A molecular model of *T. gondii* DHFR-TS complexed with JPC-2067-B was developed. We found that the three main parasite clonal types and isolates from South and Central America, the United States, Canada, China, and Sri Lanka have the same amino acid sequences preserving key binding sites for the triazine. Toxicology data are presented. JPC-2056/JPC-2067-B have potential to be more effective and less toxic treatments for toxoplasmosis than currently available medicines.
would have superb penetration into the eye and brain. These would be major advantages for this neglected disease throughout the world, and especially important in developing countries. JPC-2056/JPC-2067-B have the potential to address some of these issues (e.g. cidal for tachyzoites, less toxicity, available for oral and parenteral use, and potentially available in pediatric suspensions that are stable without refrigeration). Further testing and development will reveal whether JPC-2056/JPC-2067-B can address the other characteristics of an ideal anti-toxoplasmosis medicine.

Current treatment of toxoplasmosis includes the combination of a folic acid antagonist and an inhibitor of dihydropteroic acid synthesis. The gold standard treatment has been the classic anti-malarial combination of pyrimethamine and sulfadiazine. In vitro and in vivo experimental models of toxoplasmosis parallel this clinical approach [1,3]. Herein, results using those same in vitro and in vivo toxoplasmosis models with a new anti-malarial candidate, JPC-2067B (4, 6-diamo-no-1, 2-dihydro-2, 2-dimethyl-1-(3′, 2-chloro-4-trifluoromethoxyphenoxy) propyloxy)-1, 3, 5-triazine) and its pro-drug JPC-2056 are presented [4–10]. This new anti-malarial class [4–10], without a sulfonamide, has dramatic potency against multi-drug resistant Plasmodium falciparum strains [4–10].

We have waited a long time for a representative of this series of compounds to advance to the clinic for the treatment of T. gondii infection. This is especially important for those with this infection who are pregnant and in utero. New medications are needed because the classic gold standard medications have substantial toxicity [1,2]. Moreover, pyrimethamine cannot be used in the first trimester of pregnancy, as folate depletion is detrimental to fetal development [1]. Neutropenia is a common toxicity with pyrimethamine treatment even when leukovorin is administered in conjunction with this medicine [3]. Furthermore, pyrimethamine is generally administered in a synergistic combination with sulfadiazine which has substantial associated hypersensitivity [2] and toxicity (e.g. kidney stones or hepatic or renal complications). New medicines are greatly needed for individuals suffering from toxoplasmosis.

The extremely promising candidate, JPC-2067-B, comes from a pre-clinical anti-malarial series well known in malaria biology by the name of the related metabolite WR99210 (4,6-diamo-no-1,2-dihydro-2,2-dimethyl-1-[3′(2,4,5-trichlorophenoxy) propyloxy]-1, 3, 5-triazine) [4–10]. In vitro anti-malarial testing of WR99210 against drug-sensitive and drug-resistant strains has shown high potency and full activity against P. falciparum strains not responsive to pyrimethamine, proguanil or chloroquine with an ED₅₀ of 0.05 ng/mL in vitro. As yet there is no strain resistant to this class of compounds. WR99210 is discussed here in order to provide a common point of cross reference. Like proguanil, the new clinical candidate JPC-2056 (Figure 1) is a biguanide pro-drug which is metabolized in vivo to the active dihydrotriazine JPC-2067-B (Figure 1). For in vitro testing the metabolite must be used; for oral usage the biguanide must be given. The ongoing work in development and progression to use in the care of patients of this very promising anti-malarial clinical candidate [Jacobus et al., unpublished] also is useful in development of the same medicine for treatment of toxoplasmosis.

The previously described triazine WR99210 and its pro-drug, PS-15, were developed in response to resistance of P. falciparum to pyrimethamine and cycloguani [4–11]. WR99210 was found to be a very tight binding and potent inhibitor of P. falciparum DHFR-TS [4–11]. WR99210 and PS-15 also were highly active in vivo against P. falciparum, with activity 2 logs greater than that of pyrimethamine. These compounds were also highly active against P. vivax, without cross-resistance to other antifolates (S. Hunt, personal communication). The therapeutic/toxic ratio is increased because the high avidity of these compounds for the P. falciparum DHFR differs from its lower avidity to mammalian DHFR [11]. Unfortunately, toxicity of WR99210 limited its development and use and it will not be a clinically useful compound.

We previously evaluated the active triazine metabolite of proguanil (cycloguani) against T. gondii tachyzoites [12], and more recently found that WR99210 was also highly active against T. gondii in vitro and in vivo when administered parenterally [13]. PS15 also was found to be effective in vivo [13]. A major drug discovery effort over the past 6 years has identified an analog of WR99210, JPC-2067-B, which has superior pharmacological characteristics. Importantly, pro-drug JPC-2056, is easily absorbed, bioavailable, and relatively nontoxic. In studies with P. falciparum, oral administration of JPC-2056 resulted in conversion to the JPC-2067-B which was cidal for the malaria parasite. The high potency and selectivity of JPC-2067-B for inhibition of apicomplexan parasite DHFR relative to mammalian DHFR reduces the likelihood of neutropenia, thus enhancing the margin of safety and convenience in monitoring white blood counts with its use. JPC-2056 was also as active as monotherapy in vitro as the synergistic combination of pyrimethamine and sulfadiazine and is currently being advanced to clinical trials, leading to a new and markedly improved class of anti-folate medicines for the treatment of malaria.

The effect of JPC-2067-B on T. gondii is of considerable interest and importance. The lack of toxicity of JPC-2067-B and the favorable absorption and distribution profile of its prodrug JPC-2056 offers the possibility of overcoming the limitations of pyrimethamine. The benefit of greater specificity for the parasite rather than host DHFR could have the dual advantage of reducing host toxicity while eliminating the need for simultaneous administration of a sulfonamide. Whether an IC₅₀ of 6.5 nM is sufficient to be used as a single agent for either malaria or toxoplasmosis or would be better used in conjunction with another anti-microbial in vivo under clinical conditions remains to be determined.

Structures of JPC-2067-B and its corresponding pro-drug JPC-2056 (Jacobs Pharmaceutical Company, Princeton, NJ) are shown in Figure 1. The biguanide pro-drug is converted in vivo to the biologically active dihydrotriazine through P450 metabolism in the liver, and so in vitro experiments are always conducted with the dihydrotriazine (JPC-2067-B). The overall aim of the experiments was to determine effect of the dihydrotriazine on T.
gongii in vitro and in vivo and inhibitory effect of the dihydrotriazine on T. gondii that was observed is described herein.

Methods

Parasites and assessment of effect of inhibitors on T. gondii tachyzoites in tissue culture and cells in tissue culture

Tachyzoites of the RH strain of T. gondii were passaged in human foreskin fibroblasts (HFF). They were used to infect fibroblasts to determine antimicrobial effects of candidate compounds. Outcome was assessed with microscopy and uracil uptake after four days in culture as described [8,12,13]. Briefly, for testing of inhibitors in vitro against T. gondii tachyzoites, four-day old confluent cultures of human foreskin fibroblasts (HFF) were infected with 10^5 tachyzoites and cultured for 1 hour to allow parasite invasion. Inhibitor was added and cells cultured for 3 days. They were supplemented with 3H uracil and incubation extended for a further day, whereupon uracil incorporation into cells and thus parasite growth were assessed by liquid scintillation counting [8,12,13]. Studies were performed with inhibitors as described in [8,12,13]. Lack of toxicity for mammalian host cells was demonstrated first by visual inspection of the monolayer and by parallel concomitant evaluation of separate 3H thymidine incorporation assays by non-confluent HFF cell monolayers.

JPC-2067-B for use in in vitro (tissue culture) and in vivo studies

For in vitro studies, a stock solution of JPC-2067-B was initially dissolved in 100% dimethyl sulfoxide (DMSO) and then diluted in complete tissue culture medium (IMDM-C) [IMDM with NaHCO3 and 25 mM Hepes (Cambrex Bio Science, Walkersville, MD), 10% fetal bovine serum (Gibco, Grand Island, N.Y.), 1 x antibiotic-antimycotic solution (Cellgro, Mediatech), and 2 mM L-glutamine (Gibco). Working concentrations of JPC-2067-B were made using IMDM-C. Concentrations measured ranged from 10 to 100 nM. For certain in vivo studies, JPC-2067-B was initially dissolved in 100% DMSO and then diluted 100 fold in 1 x PBS without calcium or magnesium (Cellgro) and administered intraperitoneally (i.p.) 15 minutes following i.p. inoculation of the parasite. In other in vivo studies, the orally bioavailable pro-drug JPC-2056 (40 mg/kg/dose, bid) was administered per orally by gavage beginning one day following i.p. inoculation of the parasite.

DHFR enzyme activity and its inhibition by JPC-2067-B: Effect against T. gondii DHFR compared to P. falciparum DHFR and human DHFR

DHFR from T. gondii [43] prepared as above was also directly compared to purified recombinant P. falciparum DHFR (pDHFR) and purified recombinant human DHFR (hDHFR). The hDHFR was produced as the recombinant enzyme expressed in E. coli [41]. The sequence of the protein was identical to that predicted for the previously reported gene sequence [4]. DHFR from T. gondii was isolated directly from RH strain T. gondii grown in culture on Chinese hamster ovary cells lacking DHFR (CHO/dhfr–American Type Culture Collection 3952 CL) [42]. Organisms were introduced into a confluent monolayer and harvested when the mammalian cells were lysed. The 100,000 x g supernate was stored in liquid nitrogen. Mycobacterium avium-intracellulare used in these studies was a clinical isolate (serovar 4) from Indiana University School of Medicine, Department of Pathology. The strain was maintained on Lowenstein-Jensen slants (Baxter Scientific) grown at room temperature. To produce enzyme, the organism was grown in Middlebrook 7H-9 liquid medium at 37°C to an OD660 of 0.5 to 0.7, which took several weeks. At harvest, the bacteria were centrifuged, sonicated, and the 100,000 x g supernate was stored under liquid nitrogen until assay. These supernates contained both DHFR and dihydropteroate synthetase activity.

Rat liver DHFR was prepared from livers of female Sprague-Dawley rats. The 100,000 x g supernate was partially purified by ammonium sulfate precipitation; the 50–90% precipitate was redissolved and stored in liquid nitrogen.

The spectrophotometric assay for DHFR was optimized for temperature and concentration of substrate and cofactor for each enzyme. The standard assay contained Na phosphate buffer pH 7.4 (40.7 mM), 2-mercaptoethanol (8.9 mM), NADPH (0.117 mM), dihydrofolinic acid (0.09 mM), KCL (150 mM), and sufficient enzyme to produce a change in OD240 of 0.035/minute at 37°C. The reaction was continuously recorded for 3 minutes. Activity under these conditions was linear with enzyme concentration over a 4-fold range. The low background activity in the absence of dihydrofolinic acid was subtracted from all rates.

DHFR was assayed with several concentrations of inhibitor to produce rates ranging from 1 to 90% of the uninhibited rate. At least three concentrations were required for calculation; most curves contained five concentrations. Semi-logarithmic plots of the data gave sigmoidal curves that were fit by non-linear methods to determine the concentration yielding 50% inhibition [IC50] [Prism 4.0 (GraphPad)].

Effect against T. gondii DHFR compared with P. falciparum DHFR and human DHFR

DHFR from T. gondii [43] prepared as above was also directly compared to purified recombinant P. falciparum DHFR (pDHFR) and purified recombinant human DHFR (hDHFR). The hDHFR was produced as the recombinant plasmid [10]. The enzyme was purified following ammonium sulfate precipitation, metathexate/agarose affinity chromatography, and finally a Superdex 200 size exclusion column. The pDHFR isolation methods were those reported previously [11,44]. Pyrimethamine and JPC-2067-B were tested for activity against recombinant pDHFR, recombinant hDHFR and the T. gondii lyssate DHFR. The same buffer as used in the other assays comparing T. gondii DHFR with DHFRs from rat liver P. carinii and M. avium intracellulare was used but the maximal activity, temperature, and length of observation were adjusted for assays on the specific plate reader. The series of pDHFR and hDHFR assays were run twice for hDHFR and three times for pDHFR and the representative data are shown (see Results). The tDHFR sample was exhausted after one set of assays at a lower activity than the others (uninhibited change in OD240 of 0.004/min versus 0.02/min for the recombinant enzymes). The reaction was setup at 23°C, the plate loaded, and the OD240 recorded at 20 second intervals for 10 minutes. The first 8 minutes were used to generate linear fit slopes in Excel. Each concentration has been reported as the mean of 5 replicate reactions with the standard deviation reported as the error. Results are expressed as the percent of control activity versus log concentration of inhibitor. Prism 5.0 was used to generate curves from 12 different concentrations of inhibitor using a non-linear fit method.

Quantitation of JPC-2067-B

JPC-2067-B levels were quantitated using an HPLC system comprised of a Spectra System P4000 pump, AS300 autosampler, UV2000 detector and a ChromJet integrator. The column is a Phenomenex Synteryg MAX-RP 80A 130 x 4.6 mm, s/n 219259. Elution was effected with a gradient of Mobile Phase A (0.05% aqueous TFA) and Mobile Phase B (0.025% TFA in acetonitrile). The flow rate was 0.5 ml/min, the injection volume...
was 20 μl and the detector was set to 290 nm. Observed retention times for WR99210, PS-15 and JPC-2067-B were 9.5, 15.7 and 9.1 minutes, respectively.

*T. gondii* Infection of mice

Tachyzoites also were used to infect mice. Outbred Swiss Webster mice were bred in our specific pathogen free colony. When they were approximately 30 g, they received 10,000 RH strain tachyzoites intra-peritoneally (i.p.); numbers of parasites present in peritoneal fluid were counted four days later as described [8,13]. Mice were maintained and utilized in accordance with IACUC and NIH guidelines and approvals.

Studies of effect of peritoneal administration of JPC-2067-B on murine toxoplasmosis

JPC-2067-B was administered parenterally. In initial studies, this was given 15 minutes after i.p. infection and then each day for four days (12.5 mg/kg/day). Peritoneal parasite burden was quantitated on the fourth day after injection. Control mice received 1% DMSO in PBS.

Studies of effect of oral administration of JPC-2056 on murine toxoplasmosis

Beginning one day following infection of outbred SW mice with tachyzoites of the RH strain of *T. gondii* mice received JPC-2056 by gavage at a concentration of 40 mg/kg in 0.5 ml twice daily. Peritoneal *T. gondii* burden was determined on day 4 following infection.

PCR and bioinformatics of DHFR in various clonal and atypical strains of *T. gondii*

Sequences of DHFR in each of the conventional parasite clonal types (RH, type I; Me49 type II; and VEG, type III) from the data base and by PCR using strains (isolates) from Brazil, Guyana, Guatemala, Canada, China, and Sri Lanka [45–50] were determined with PCR using cDNA or g DNA as template. The primers used were: Forward, 5’-AGGGACGGTGAAGTTTCGCTTTA-3’; Reverse, 5’-TTTCCGGTCTTTCTCGCTCATCCA-3’.

Modeling of DHFR

Modeling of the *T. gondii* DHFR was based upon the crystal structure of the closely related *P. falciparum* DHFR in complex with WR99210, NADPH and dUMP (pdb id 1j3b), using the structure based sequence alignment as a guide. Those residues which displayed sequence variation between *P. falciparum* and *T. gondii* DHFR and were located within 4 Å of the ligand binding pocket were analysed to look for significant differences.

Toxicology studies

A 42-day toxicology study in CD-1 mice at doses up to 98 mg/kg evaluating well-being, weight gain, and histopathology was performed. A comparable 42-day toxicology study in CD-1 mice at doses up to 100 mg/kg evaluating well-being, weight gain, and histopathology was performed. A comparable 42-day toxicology study in CD-1 mice at doses up to 98 mg/kg evaluating well-being, weight gain, and histopathology was performed. A comparable 42-day toxicology study in CD-1 mice at doses up to 98 mg/kg evaluating well-being, weight gain, and histopathology was performed. A comparable 42-day toxicology study in CD-1 mice at doses up to 98 mg/kg evaluating well-being, weight gain, and histopathology was performed. A comparable 42-day toxicology study in CD-1 mice at doses up to 98 mg/kg evaluating well-being, weight gain, and histopathology was performed.

Lack of toxicity of JPC-2067-B for human fibroblasts tested concomitantly with *T. gondii* tachyzoites

Human foreskin fibroblasts were tested concomitantly with *T. gondii* tachyzoites with increasing concentrations of JPC-2067-B. Data from a representative experiment are also shown in Figure 2A and demonstrate no toxicity measured as uptaked of tritiated thymidine by nonconfluent fibroblasts. The increased uptake of thymidine in these cultures remains unexplained but also has been noted with certain other compounds such as triclosan.

JPC-2067-B is cidal for *T. gondii*

In separate experiments, to determine whether JPC-2067-B would be cidal for *T. gondii*, cultures were maintained for 52 days after removing JPC-2067-B on the 48th day of culture. No plaques or growth of parasites were detected (Figure 2D). The absence of growth following removal of JPC-2067-B from HFF exposed to *T. gondii* indicates that this compound is “cidal” and not merely “static” for *T. gondii*.

In vivo effect of parenteral administration of JPC-2067-B and oral administration of the pro-drug JPC-2056 on toxoplasmosis

JPC-2067-B was also highly effective against *T. gondii* tachyzoites in a mouse model. A representative experiment with JPC-2067-B is shown in Figure 3A. In the experiment in Figure 3A, mice were infected i.p. with 10,000 tachyzoites of the RH strain of *T. gondii* for 15 minutes prior to initial treatment with JPC-2067-B. For these parenterally treated mice, female mice received a dose of 1.25 mg/kg/day of JPC-2067-B, administered i.p. for the next 5 days. Control mice received an equivalent amount of DMSO (1%) in 1× PBS. In a separate experiment, DMSO at this concentration was shown not to modify subsequent parasite numbers when compared with i.p. inoculation of PBS. Mice treated with JPC-2067-B appeared sleek and active 4 days after infection. In contrast, infected control mice appeared ill, with ruffled fur and hunched posture. Intrapерitoneal parasite numbers were reduced by two logs with treatment with JPC-2067-B on the fifth day after injection of parasites (Figure 3A). These differences between control and treated mice were statistically significant (p<0.05).

In addition, a similar experiment was performed with oral administration of the orally bioavailable pro-drug JPC-2056 (40 mg/kg/dose, bid) beginning one day following i.p. inoculation of the parasite. Parasite number in peritoneal fluid was quantitated three days after that, i.e. the fourth day following infection. For the mice orally treated with JPC-2056 there were similar significant differences in parasite peritoneal burden on the third day of treatment (Figure 3B, p<0.03).
Figure 2. Effect of JPC-2067-B on *T. gondii* in human foreskin fibroblasts. A. Thymidine uptake assay demonstrates no toxic effect on host cells. B. Uracil uptake assay demonstrates that JPC-2067-B is effective against *T. gondii* at low nanomolar concentrations. C. Micrographs showing marked inhibition of *T. gondii* by JPC-2067-B. Note absence of plaques and parasites in treated cultures. Concentrations prepared are shown. D. Micrograph showing absence of destruction of monolayers infected, exposed to JPC-2067-B for 4 days and cultured for prolonged times with *T. gondii*. The control monolayer was completely destroyed by 5 days of culture. This contrasts with similarly infected monolayers in the micrograph exposed to JPC-2067-B for 4 days and then with the JPC-2067-B removed. No plaques or *Toxoplasma* were seen throughout 52 days of culture, demonstrating that the JPC-2067-B is cidal and no drug resistant mutants were selected in this experiment. No plaques were present from 4 days through the subsequent 1 and 1/2 months after *in vitro* challenge.

doi:10.1371/journal.pntd.0000190.g002
Effect of JPC-2067-B on *T. gondii* DHFR enzyme activity compared with effects on DHFR enzyme activity from mammalian cells, *Pneumocystis carinii*, and *Mycobacterium avium-intercellulare*

The IC50 values determined for reference compound pyrimethamine (JPC-1090) were in agreement with prior assays of the compound (S. Queener, unpublished data). Both JPC-1090 and JPC-2013 (cycloguanil) had IC50 values in the micromolar range and were not significantly selective for pathogen DHFR (Table 1). JPC-208 (WR92210) was more potent, with IC50 values in the nanomolar range, but was not selective. JPC-2067-B had nanomolar IC50 values for the DHFRs from all three pathogens and higher IC50 value for the mammalian DHFR, yielding about 3.4 to 5.9 fold selectivity. The potency for this compound greatly exceeds the concentration of pyrimethamine used clinically (Figure 4).

Semilogarithmic plots of the data yielded normal sigmoidal curves for pyrimethamine and cycloguanil (Hill slope of the

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**Figure 3. Reduction of numbers of parasites in peritoneal fluid.**

A. Reduction of numbers by i.p. treatment of mice with JPC-2067-B. B. Reduction of numbers of parasites following treatment of mice with the pro-drug JPC-2056. JPC-2056 was administered by gavage. JPC-2056 is converted into the active compound, JPC-2067-B. doi:10.1371/journal.pntd.0000190.g003
normalized log-concentration-response curve was about $-1$ but both WR-99210 and JPC-2067-B yielded very steep curves for the DHFRs from rat liver, P. carinii, and T. gondii; these compounds produced normal dose response curves with M. avium DHFR. The steep Hill slopes for JPC-208 and JPC-2067-B suggests that the interaction of these compounds with these enzymes is not following a simple 1:1 interaction expected with a competitive inhibitor.

**Table 1.** Comparison of IC50 of JPC-2067-B against DHFRs of T. gondii and other opportunistic pathogens that are harmful to patients with AIDS.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Rat liver DHFR IC50 (µM)</th>
<th>Pc DHFR IC50 (µM)</th>
<th>Tg DHFR IC50 (µM)</th>
<th>Mav DHFR IC50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JPC-1090-A-1 (Pyrimethamine)</td>
<td>1.32 (1.14–1.55)</td>
<td>6.17 (5.45–6.98)</td>
<td>0.14 (0.12–0.16)</td>
<td>1.2 (0.93–1.45)</td>
</tr>
<tr>
<td>JPC-2013-B-1</td>
<td>1.73 (1.48–2.03)</td>
<td>8.9 (8.2–9.7)</td>
<td>0.41 (0.38–0.43)</td>
<td>12.1 (11.2–13.1)</td>
</tr>
<tr>
<td>JPC-208-B (WR99210)</td>
<td>0.00081 (0.0007–0.00093)</td>
<td>0.00265 (0.00025–0.00029)</td>
<td>0.000602 (0.00057–0.00063)</td>
<td>0.00057 (0.00047–0.00071)</td>
</tr>
<tr>
<td>JPC-2067-B</td>
<td>0.0222 (0.0206–0.0238)</td>
<td>0.00393 (0.00371–0.00417)</td>
<td>0.0065 (0.00626–0.00674)</td>
<td>0.00375 (0.00291–0.00483)</td>
</tr>
</tbody>
</table>

Values shown are micromolar IC50 values (95% confidence limits). doi:10.1371/journal.pntd.0001900.t001

In order to further investigate the efficacy of the dihydrotriazines on P. falciparum versus T. gondii with regard to drug design and molecular mode of action, we have analyzed structural models of the DHFR enzyme in the apicomplexan parasites. Of the 9 residues which form interactions with the dihydrotriazine inhibitor, WR99210 in the structure of P. falciparum DHFR Ile14, Cys15, Asp54, Met55, Phe58, Ile111, Leu119, Ile164 and Tyr170 all are either identical or very similar in T. gondii DHFR (Figure 6A). In particular Asp54 and Tyr170 which make important H-bonds to the inhibitor are conserved in T. gondii DHFR. Furthermore, modeling studies suggest that the substitution of Ile in P. falciparum DHFR for Met and Val at positions 111 and 164, respectively, in T. gondii DHFR results in little change in the Van der Waals packing interactions made to the inhibitor (Figure 6B). Modeling of the potent inhibitor JPC-2067-B into T. gondii DHFR reveals that the additional trifluoromethoxy group is positioned such that it is exposed to the solvent and as such can probably be tolerated by the enzyme with respect to inhibitor.

**Effect of JPC-2067-B on T. gondii DHFR enzyme activity compared with effect on P. falciparum and human DHFR enzyme activities**

In Figure 5 and Table 2, the IC50 for P. falciparum DHFR was 3.9 nM, T. gondii DHFR was 32 nM, and human DHFR was 150 nM. For pyrimethamine, the IC50 for P. falciparum was 42 nM, for T. gondii DHFR was 280 nM, and for human DHFR was 1,900 nM.

The differences in values between Figures 4 and 5 may be due to variations in assays. Assays towards the comparison to inhibition of opportunistic pathogens are run on partially purified lysates at 37°C for a shorter duration while this set of assays is run in a high-throughput manner with several recombinant enzymes and a more drawn out observation time at 23°C. The amount of enzyme used has been reduced to extend the length of observation and minimize the effect of data points lost during plate setup. These differences in methodology likely explain the slight shift in IC50. The ratio of IC50 values measured via high throughput method (hDHFR/tgDHFR) to the ratio measured from lysates (rat liver DHFR/TgDHFR) under different conditions are 4.6 versus 3.4, which are comparable.

Overall, JPC-2067-B has considerable potency and some selectivity relative to mammalian reference enzymes, in two independent laboratories under slightly different assay conditions demonstrating the effect of this compound on T. gondii DHFR.

**Molecular modeling of T. gondii DHFR [51] and JPC-2067-B**

In order to further investigate the efficacy of the dihydrotriazines on P. falciparum versus T. gondii with regard to drug design and molecular mode of action, we have analyzed structural models of the DHFR enzyme in the apicomplexan parasites. Of the 9 residues which form interactions with the dihydrotriazine inhibitor, WR99210 in the structure of P. falciparum DHFR Ile14, Cys15, Asp54, Met55, Phe58, Ile111, Leu119, Ile164 and Tyr170 all are either identical or very similar in T. gondii DHFR (Figure 6A). In particular Asp54 and Tyr170 which make important H-bonds to the inhibitor are conserved in T. gondii DHFR. Furthermore, modeling studies suggest that the substitution of Ile in P. falciparum DHFR for Met and Val at positions 111 and 164, respectively, in T. gondii DHFR results in little change in the Van der Waals packing interactions made to the inhibitor (Figure 6B). Modeling of the potent inhibitor JPC-2067-B into T. gondii DHFR reveals that the additional trifluoromethoxy group is positioned such that it is exposed to the solvent and as such can probably be tolerated by the enzyme with respect to inhibitor.
binding. In addition, Cys50, which when mutated has been shown to play a role in pyrimethamine resistance [52] in *P. falciparum* DHFR, is replaced by His27 in *T. gondii* DHFR. Given its position close to the trifluoromethoxy group of JPC-2067-B it may well be that further modification to this part of the inhibitor could lead to favorable interactions with the imidazole ring of His27 (Figure 6B). However, these small changes in the *T. gondii* JPC-2067-B binding site, when compared to its homologue in *P. falciparum* may contribute to the somewhat lower sensitivity of this enzyme to JPC-2067-B.

DHFR sequences from clonal type I, II and III parasites and atypical parasites including those found in developing tropical countries

The deduced amino acid sequences of DHFRs [43] in the data base for RH(U.S., type I), Me49 (U.S., type II), VEG (U.S., type III), and Goug (atypical), and identified by PCR of DHFRs from strains isolated from Brazil, Canada, Guyana, Guatemala, China and Sri Lanka (Table 3, [45–50]) were identical (data not shown).
Parasites, i.e., from clonal types I, II and III and atypical strains, are a possibility. To begin to address this issue as it is relevant to toxoplasmosis in the developing world, we have compared the sequences of DHFRs in each of the conventional parasite clonal types (I, II, and III) from the data base, and by PCR of DHFR and analyses of isolates from Brazil, Guyana, Guatemala, China, and Sri Lanka [45–50] demonstrates that the key amino acids for binding the triazine are conserved (data not shown). There are parasites that are genetically different in different countries, e.g. in Brazil there are a variety of genetically different parasites of clonal type I/III background with an association with a very high prevalence of retinal disease; in Northern Coastal South America highly virulent parasites that have recently been lethal or caused severe illness and death in French soldiers in French Guiana and in a recent epidemic in a village in Suriname [53]; and atypical parasites in Central America and Mexico. In Asia there are unique genotypes which differ from the typical I, II, III genotypes, and in Africa there are all the genetic clonal types of parasites. In Europe and Poland the predominant type is clonal type II, and in the U.S. there are other types but a recent abstract described predominance of type II parasites. In an epidemic in Sea Otters in Moro Bay California and on Vancouver Island, the parasites are also atypical. Each of these parasites might have different growth rates (new isolates often grow more slowly than laboratory adapted strains, JP Dubey, personal observations) and DHFRs with slightly different sequences or significant mutations are a possibility. To begin to address this issue as it is relevant to toxoplasmosis in the developing world, we have compared the sequences of DHFRs in each of the conventional parasite clonal types (I, II, and III) from the database, and by PCR of DHFR from isolates including a Brazilian strain, a strain from Guyana, a strain from Guatemala, a strain from Canada, a strain from China and a strain from Sri Lanka (Table 3; [45–50]). There are no differences in amino acid sequence of the DHFRs.

As shown in the enzyme inhibition and parasite inhibition assays herein, upon conversion of JPC-2056 to JPC-2067-B by cytochrome p 450, the product, JPC-2067-B, becomes a highly effective treatment for apicomplexan infections. Toxicological data (Table 4) supports the advancement of JPC-2056 to clinical development. A 42-day toxicity study in CD-1 mice produced no histopathology findings at doses up to 98 mg/kg and no gross pathology with the exception of a reduction in the rate of weight gain. A comparable 42-day toxicity study produced histopathology findings at 15 mg/kg with sporadic episodes of loose stools/diarrhea that resolved upon drug withdrawal. There was no histopathology or gastrointestinal effects observed in the 42-day period. 7.5 mg/kg was established as the NOAEL (No Observed Adverse Effect Level). When JPC-2056 and JPC-2067 were assayed in the Ames Test, with and without microsomal activation, no activity was exhibited with tester strains TA97, TA98, TA100, TA102 and TA1535 (Table 4).

### Table 2. Comparison of IC$_{50}$ of JPC-2067-B and Pyrimethamine against recombinant PfDHFR and hDHFR and DHFR activity of T. gondii lysates.

<table>
<thead>
<tr>
<th>Compound</th>
<th>hDHFR IC$_{50}$ (µM)</th>
<th>PfDHFR IC$_{50}$ (µM)</th>
<th>TgDHFR IC$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JPC-2056-A-1 (Pyrimethamine)</td>
<td>1.90 (1.77–2.05)</td>
<td>0.0422 (0.0369–0.0483)</td>
<td>0.275 (0.236–0.321)</td>
</tr>
<tr>
<td>JPC-2067-B</td>
<td>0.148 (0.136–0.162)</td>
<td>0.00388 (0.00364–0.00414)</td>
<td>0.0320 (0.0245–0.0417)</td>
</tr>
</tbody>
</table>

Values shown are micromolar IC$_{50}$ values (95% Confidence Intervals).

doi:10.1371/journal.pntd.0001190.t002

**Toxicology studies**

A 42-day toxicity study in CD-1 mice (Table 4) produced no histopathology findings at doses up to 98 mg/kg and no gross pathology with the exception of a reduction in the rate of weight gain. A comparable 42-day toxicity study in *Macaca fascicularis* (Table 4) produced histopathology findings at 15 mg/kg with sporadic episodes of loose stools/diarrhea that resolved upon drug withdrawal. There was no histopathology or gastrointestinal effects observed in the 42-day period. 7.5 mg/kg was established as the NOAEL (No Observed Adverse Effect Level). When JPC-2056 and JPC-2067 were assayed in the Ames Test, with and without microsomal activation, no activity was exhibited with tester strains TA97, TA98, TA100, TA102 and TA1535 (Table 4).

### Discussion

Our studies demonstrate that JPC-2067-B is effective against *T. gondii* in *vitro* with an IC50 of 20 nM and in *vivo* when administered by i.p. injection and the pro-drug JPC-2056 is effective *in vitro* when administered orally. Each of our results described herein with this novel new class of anti-folate compound, dihydrotriazine, parallels earlier findings with progenitors of this class which were not as suitable for use for humans, e.g. proguanil [12] and WR99210 [13]. The major and compelling advantages of JPC-2056, which is moving into clinical trials, is in the reduction of toxicity and development of a much more readily bioavailable compound than WR99210. WR99210 will never be a medicine for humans because of difficulties in those areas, also reflected in the effect on the mammalian enzyme, Table 1. The advantages of bioavailability, high potency, specificity, selectivity and potential for elimination of toxicities that occur with pyrimethamine either used alone or in conjunction with sulfadiazine and other medicines and because JPC-2056 will be entering clinical trials for the treatment of malaria, testing of this new class of anti-folates against the related apicomplexan *T. gondii*, was very important. Our results suggest that the activity against *T. gondii* is significant and that JPC-2056 has the potential to replace the combination of pyrimethamine plus sulfadiazine or second line drugs in the treatment of toxoplasmosis.

The modeling of *T. gondii* DHFR in complex with this family of inhibitors gives us understanding at the molecular level of why compounds of this class are highly active against *T. gondii* tachyzoites. JPC-2056 already has been optimized for pharmacokinetics and lack of toxicity and is being progressed to the clinic as a potentially effective treatment for both *P. falciparum* and *P. vivax* malaria. This ongoing work with malaria treatment provides a major benefit for the development of JPC-2056 for the treatment of toxoplasmosis.

It was of importance to determine whether in DHFR the amino acids that bind this novel, highly active triazine vary in any of the atypical parasites. Analysis of available DHFR sequences in the database for *T. gondii* isolates called RH, Me 49, VEG and Cough parasites, i.e., from a clonal types I, II and III and atypical strains, and analyses of isolates from Brazil, Guyana, Guatemala, Canada, China, and Sri Lanka [45–50] demonstrates that the key amino acids for binding the triazine are conserved (data not shown). There are parasites that are genetically different in different countries, e.g. in Brazil there are a variety of genetically different parasites of clonal type I/III background with an association with a very high prevalence of retinal disease; in Northern Coastal South America highly virulent parasites that have recently been lethal or caused severe illness and death in French soldiers in French Guiana and in a recent epidemic in a village in Suriname [53]; and atypical parasites in Central America and Mexico. In Asia there are unique genotypes which differ from the typical I, II, III genotypes, and in Africa there are all the genetic clonal types of parasites. In Europe and Poland the predominant type is clonal type II, and in the U.S. there are other types but a recent abstract described predominance of type II parasites. In an epidemic in Sea Otters in Moro Bay California and on Vancouver Island, the parasites are also atypical. Each of these parasites might have different growth rates (new isolates often grow more slowly than laboratory adapted strains, JP Dubey, personal observations) and DHFRs with slightly different sequences or significant mutations are a possibility. To begin to address this issue as it is relevant to toxoplasmosis in the developing world, we have compared the sequences of DHFRs in each of the conventional parasite clonal types (I, II, and III) from the database, and by PCR of DHFR from isolates including a Brazilian strain, a strain from Guyana, a strain from Guatemala, a strain from Canada, a strain from China and a strain from Sri Lanka (Table 3; [45–50]). There are no differences in amino acid sequence of the DHFRs.

As shown in the enzyme inhibition and parasite inhibition assays herein, upon conversion of JPC-2056 to JPC-2067-B by cytochrome p 450, the product, JPC-2067-B, becomes a highly effective treatment for apicomplexan infections. Toxicological data (Table 4) supports the advancement of JPC-2056 to clinical development. A 42-day toxicity study in CD-1 mice produced no histopathology findings at doses up to 98 mg/kg and no gross pathology with the exception of a reduction in the rate of weight gain. A comparable 42-day toxicity study in *Macaca fascicularis* produced histopathology findings at 15 mg/kg with sporadic episodes of loose stools/diarrhea that resolved upon drug withdrawal. Antimicrobial activities of JPC-2067 suggest that the gastrointestinal events may be related to disruptions in intestinal flora. No histopathology or gastrointestinal effects were observed over the 42-day period. 7.5 mg/kg was established as the NOAEL (No Observed Adverse Effect Level). JPC-2056 and JPC-2067 were assayed in the Ames Test with and without microsomal activation. No activity was exhibited with tester strains TA97, TA98, TA100, TA102 and TA1535. In summary, JPC-2056 has two advantages over WR99210. Biguanides are better absorbed and less toxic than their dihydrotriazine metabolites as has been well established in the case of Proguanil. Cycloguanil, the active dihydrotriazine metabolite of biguanide prodrug Proguanil is...
Figure 6. Structure based sequence alignment of DHFR-TS and stereo view of DHFR/NADPH/WR99210 complex inhibitor binding site. A. A structure based sequence alignment of the DHFR-TS enzymes from *P. falciparum* (*P. fal*), *P. gallinaceum* (*P. gal*), *A. thaliana* (*A. tha*), *Medicago truncatula* (*M. tru*), *Theileria annulata* (*T. ann*) and *T. gondii* (*T. gon*). The sequence numbering for the *P. falciparum* and *T. gondii* is given above and below the alignment, respectively. The secondary structure elements for *P. falciparum* DHFR-TS are given above the alignment with blue cylinders and red arrows representing α-helices and β-sheets, respectively. Residues which display sequence conservation across all species are highlighted by a black box with reverse type. Those residues which are involved in binding NADPH, pyrimethamine and WR99210 are highlighted by a red, blue and green box below the alignment, respectively, with those residues which bind both inhibitors and/or NADPH displayed with multiple colored boxes. B. A stereo view of the *P. falciparum* DHFR/NADPH/WR99210 complex inhibitor binding site with the closely related inhibitor JPC-2067-B modeled. Those residues which form close interactions with the inhibitor are labeled and shown in a stick format, colored yellow, red, blue and orange for carbon, oxygen, nitrogen and sulfur, respectively. The modeled JPC-2067-B inhibitor and NADPH cofactor are colored purple, blue, red, orange, cyan and green for carbon, nitrogen, oxygen, phosphorous, fluorine and chlorine, respectively. In addition His27, in *T. gondii* DHFR, which replaces Cys50 in *P. falciparum* DHFR, is also shown in a stick format (colored orange for carbon and blue for nitrogen) to demonstrate its close proximity to the modeled JPC-2067-B inhibitor.

doi:10.1371/journal.pntd.0000190.g006
poorly absorbed and is locally toxic. In addition, WR99210 and its biguanide PS-15 possess a 2,4,5-trichlorophenoxy structural feature, which is synthesized from 2,4,5-trichlorophenol. This phenol has the potential to generate the highly regulated toxin, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). This issue precluded the development of both WR99210 as well as its prodrug PS-15. Significantly, JPC-2056 and its active metabolite JPC-2067-B are devoid of this liability and as such offer a significant advance in this therapeutic class (Table 4). Finally, the LD50 value for JPC-2056 at high doses in the Thompson Antimalarial Assay provides sufficient therapeutic index to justify continued clinical development.

Improved, simpler to use, less toxic drugs that are easily affordable, which can be prepared in stable solution, are needed to treat toxoplasmosis. This new biguanide, moving into clinical trials, promises to be a major advance for the treatment of those with all forms of toxoplasmosis throughout the world. The development of JPC-2056 addresses factors limiting use of current medicines in the developing world for this neglected tropical disease including ease of administration, lack of toxicity, ease of monitoring, the potential for low cost, pediatric and parenteral formulations of a new and improved medicine. This therapeutic is likely to be of special benefit for those with this neglected tropical disease in developing countries.

Table 3. Parasites from Brazil, Guyana, Guatemala, Canada, China and Sri Lanka that were sources of DHFR analyzed.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Country</th>
<th>Source</th>
<th>SAG2</th>
<th>Reference: author(date), Reference number</th>
</tr>
</thead>
<tbody>
<tr>
<td>TgCxGa1</td>
<td>Guatemala</td>
<td>Chicken</td>
<td>III, nd</td>
<td>Dubey et al. (2005) [50]</td>
</tr>
<tr>
<td>TgCxBr125</td>
<td>Brazil</td>
<td>Chicken30</td>
<td>III, nd</td>
<td>Dubey et al. (2006) [49]</td>
</tr>
<tr>
<td>TgCIPRC7</td>
<td>China</td>
<td>Cat21</td>
<td>II, nc</td>
<td>Dubey et al. (2007a) [48]</td>
</tr>
<tr>
<td>TgCgG2</td>
<td>Guyana</td>
<td>Chicken4</td>
<td>III, nc</td>
<td>Dubey et al. (2007b) [46]</td>
</tr>
<tr>
<td>TgRcCa2</td>
<td>Canada</td>
<td>Raccoon3</td>
<td>II, nc</td>
<td>Dubey et al. (2008) [45]</td>
</tr>
<tr>
<td>TgDgS12</td>
<td>Sri Lanka</td>
<td>Dog31</td>
<td>II, nc</td>
<td>Dubey et al. (2007c) [47]</td>
</tr>
</tbody>
</table>

nc = non clonal, 10 markers were used and data are in references numbers; the 10 nuclear markers include SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1 and a new SAG2, and an apicoplast marker Apico.

nd = only SAGII data.
doi:10.1371/journal.pntd.0000190.t003

Table 4. Structural and Therapeutic Comparisons of JPC-2067, JPC-2056, WR99210 and PS-15.

<table>
<thead>
<tr>
<th></th>
<th>JPC-2067</th>
<th>JPC-2056</th>
<th>WR99210</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structural Class</td>
<td>Dihydrotriazine</td>
<td>Biguanide</td>
<td>Dihydrotriazine</td>
</tr>
<tr>
<td>Manufacturing Liabilities</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>TEST SYSTEM</td>
<td>TOXICOLOGICAL FINDINGS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Ames w/o Activation</td>
<td>Not Active*</td>
<td>Not Active*</td>
<td>Not Active*</td>
</tr>
<tr>
<td>1 Ames with Activation</td>
<td>Not Active*</td>
<td>Not Active*</td>
<td>Not Active*</td>
</tr>
<tr>
<td>1 CD-1 Mice</td>
<td>Inhibition of Weight Gain at 98 mg/kg for 42 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Cynomolgous Monkey</td>
<td>Intermittent Loose Stools/Diarrhea 15 mg/kg for 42 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Oral LD50 Rat</td>
<td>1,980 mg/kg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Oral LD50 Mouse</td>
<td>3,510 mg/kg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Thompson Assay LD50</td>
<td>256 mg/kg @ Day 6</td>
<td>&gt;128 mg/kg @ Day 6</td>
<td></td>
</tr>
</tbody>
</table>

*Tester Strains TA97, TA98, TA100, TA102 and TA1535.
#Tester Strains TA98, TA100, TA1535, TA1537 and TA1538.
doi:10.1371/journal.pntd.0000190.t004