AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

EXPERIMENTAL THERAPEUTICS

Efficacy of Guanabenz Combination Therapy against Chronic Toxoplasmosis across Multiple Mouse Strains

Jennifer Martynowicz,[a] J. Stone Doggett,[c,d] William J. Sullivan, Jr.[a,b]

[a]Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, Indiana, USA
[b]Department of Pharmacology and Toxicology, Indiana University School of Medicine, Indianapolis, Indiana, USA
[c]Division of Infectious Diseases, Oregon Health and Science University School of Medicine, Portland, Oregon, USA
[d]Department of Hospital and Specialty Medicine, Veterans Affairs Portland Health Care System, Portland, Oregon, USA

ABSTRACT *Toxoplasma gondii*, an obligate intracellular parasite that can cause life-threatening acute disease, differentiates into a quiescent cyst stage to establish life-long chronic infections in animal hosts, including humans. This tissue cyst reservoir, which can reactivate into an acute infection, is currently refractory to clinically available therapeutics. Recently, we and others have discovered drugs capable of significantly reducing the brain cyst burden in latently infected mice, but not to undetectable levels. In this study, we examined the use of novel combination therapies possessing multiple mechanisms of action in mouse models of latent toxoplasmosis. Our drug regimens included combinations of pyrimethamine, clindamycin, guanabenz, and endochin-like quinolones (ELQs) and were administered to two different mouse strains in an attempt to eradicate brain tissue cysts. We observed mouse strain-dependent effects with these drug treatments: pyrimethamine-guanabenz showed synergistic efficacy in C57BL/6 mice yet did not improve upon guanabenz monotherapy in BALB/c mice. Contrary to promising *in vitro* results demonstrating toxicity to bradyzoites, we observed an antagonistic effect between guanabenz and ELQ-334 *in vivo*. While we were unable to completely eliminate the brain cyst burden, we found that a combination treatment with ELQ-334 and pyrimethamine impressively reduced the brain cyst burden by 95% in C57BL/6 mice, which approached the limit of detection. These analyses highlight the importance of evaluating anti-infective drugs in multiple mouse strains and will help inform further preclinical studies of cocktail therapies designed to treat chronic toxoplasmosis.

KEYWORDS *Toxoplasma*, antimicrobial combinations, drug development, guanabenz, host-pathogen interactions, parasites

Toxoplasma gondii is an obligate intracellular parasite of warm-blooded vertebrates, and it has infected up to one-third of humans (1). The parasite can be transmitted across three life cycle stages: proliferative tachyzoites can cross the placenta and infect the fetus, latent bradyzoites can be consumed in raw or undercooked meat, and oocysts excreted by the definitive host (felines) can be ingested or inhaled (2). The destructive lytic nature of tachyzoites can cause widespread damage throughout the host, and infection with tachyzoites is lethal without treatment (3). However, in an immunocompetent host, tachyzoites rapidly convert into bradyzoites, which reside in intracellular tissue cysts that form in multiple muscle and organ systems, including the brain (4–6). The bradyzoite-containing tissue cysts are responsible for long-lasting chronic infection.

Upon immunosuppression of the host, bradyzoites convert into the lytic tachyzoites and cause acute disease. Individuals who are at risk for reactivation of acute infection are placed on prophylaxis until they can return to immune competency. For hemato-

Citation Martynowicz J, Doggett JS, Sullivan WJ, Jr. 2020. Efficacy of guanabenz combination therapy against chronic toxoplasmosis across multiple mouse strains. Antimicrob Agents Chemother 64:e00539-20. https://doi.org/10.1128/AAC.00539-20.

Address correspondence to William J. Sullivan, Jr., wjsulliv@iu.edu.

For a companion article on this topic, see https://doi.org/10.1128/AAC.00535-20.

Received 19 March 2020
Returned for modification 4 May 2020
Accepted 2 June 2020

Accepted manuscript posted online 15 June 2020
Published 20 August 2020

poietic cell transplant patients and solid organ transplant patients who develop toxoplasmosis, lifelong secondary prophylaxis may be required (7). The current frontline treatment with antifolates (pyrimethamine-sulfadiazine) suffers serious shortcomings (8). Up to a third of patients are allergic to sulfa drugs, and there is a high rate of adverse effects in response to pyrimethamine, most notably, hematologic toxicity. These adverse events are noted in up to 60% of *Toxoplasma* encephalitis patients, which leads to the discontinuation of therapy in up to 45% of patients (3, 9, 10). Additional side effects include leukopenia, thrombocytopenia, cutaneous rash, and fever (10). Most importantly, no approved treatment effectively targets tissue cysts. It is therefore of clinical importance to identify better-tolerated agents that can eliminate the tissue cyst reservoir that constantly jeopardizes the life of susceptible individuals.

Targeting of tissue cysts remains a technical challenge due to their quiescent nature and proclivity for the brain. Experimental drug therapies are usually tested in mice, and a reduction of the quantity of brain cysts is presumed to be representative of cyst reduction throughout the body. Only in the last decade have a few compounds capable of significantly reducing tissue cysts *in vivo* appeared. Treatment with compound 32, which was optimized for use against *T. gondii* calcium-dependent protein kinase 1 (TgCDPK1), showed an 88.7% decrease in the brain cyst burden in BALB/c mice (11). Endochin-like quinolones (ELQs), which target the parasite cytochrome bc_1 complex, have also shown promise in lowering cyst counts in mouse models (12, 13). ELQ-316, in particular, reduces the brain cyst burden 76% to 88% in CBA/J mice (14).

Previous work in the Sullivan lab found that the FDA-approved antihypertensive drug guanabenz can be repurposed as an antiparasitic (15–17). As reported in other eukaryotic cells, guanabenz can inhibit the dephosphorylation of the translation initiation factor α subunit of eukaryotic initiation factor 2 in *Toxoplasma*, which is a key switch associated with stage conversion (15). We have shown that guanabenz inhibits the growth of tachyzoites and reduces the brain cyst burden in chronically infected BALB/c mice 65% to 80% (16). Interestingly, guanabenz had an unexpected effect on pathogenesis in C57BL/6 mice, which are genetically more susceptible to *Toxoplasma* infection than BALB/c mice (17–19). Half of the chronically infected mice treated with guanabenz died, and the cyst burden increased in the surviving C57BL/6 mice. The efficacy of compound 32 and ELQs in the more susceptible mouse strain, C57BL/6, has not been determined.

A common shortfall of these three cyst-reducing compounds is that none achieve a radical cure; for unknown reasons, they are unable to completely eliminate *Toxoplasma* from the brain. Eradicating this residual cyst population is not a simple matter of prolonging the treatment time. We have previously attempted to prolong guanabenz treatment to 6 weeks but were unable to improve the cyst burden reduction (17). Additionally, a residual population of cysts was still maintained after prolonged treatment with ELQs (20).

While the reduction in brain cysts with these novel compounds is promising, the ideal treatment needs to eliminate them completely. In this study, we tested the hypothesis that combination treatments coupling guanabenz with various drugs possessing different mechanisms of action would lower the cyst burden beyond that achieved with monotherapy.

RESULTS

Pyrimethamine prevents guanabenz-associated death in C57BL/6 mice and reduces the brain cyst burden. We previously showed that while guanabenz effectively reduces the brain cyst burden in BALB/c mice, it increases the cyst burden in C57BL/6 mice (17). The C57BL/6 mice also developed symptoms of *Toxoplasma* encephalitis during guanabenz treatment, resulting in the loss of half the treatment group. We postulated that the guanabenz treatment led to reactivation of latently encysted parasites in C57BL/6 mice. In an attempt to prevent the death of chronically infected C57BL/6 mice treated with guanabenz, we coadministered pyrimethamine; pyrimethamine, which specifically targets replicating parasites, is the frontline antifo-

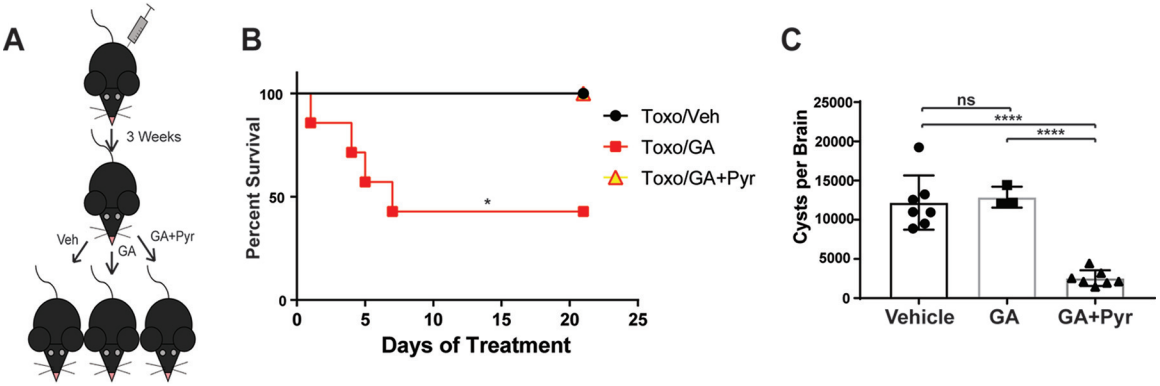

FIG 1 Pyrimethamine combination therapy in C57BL/6 mice. (A) Six-week old, female C57BL/6 mice were infected intraperitoneally with *Toxoplasma* (gray syringe) and allowed to progress to chronic infection for 3 weeks. The mice were then randomized into three different treatment groups ($n = 7$). (B) The latently infected mice were treated for 3 weeks, and survival was tracked over that time. *, $P = 0.0221$, log-rank test. (C) At the conclusion, the brain tissue cyst burden was quantified in a blind manner for each mouse. *P* values were determined by one-way ANOVA, followed by an unpaired Student *t* test. ns, not significant; ****, $P < 0.0001$. Toxo, *Toxoplasma* infected; Veh, vehicle; GA, guanabenz; Pyr, pyrimethamine.

late used to treat acute toxoplasmosis (Fig. 1A). As seen previously (17), treatment of latently infected C57BL/6 mice with guanabenz alone resulted in the death of half the group (Fig. 1B). In contrast, none of the infected C57BL/6 mice coadministered guanabenz and pyrimethamine perished (Fig. 1B). The efficacy of pyrimethamine with guanabenz suggests that the detrimental effects of guanabenz treatment in C57BL/6 mice can be nullified with combination therapy.

At the conclusion of the study, the brains were collected and the cyst burden was quantified in a blind manner. As seen before (17), guanabenz alone failed to reduce the cyst counts in the brains of C57BL/6 mice (Fig. 1C). Surprisingly, combination therapy with guanabenz and pyrimethamine restored the cyst-lowering capabilities of guanabenz in C57BL/6 mice (Fig. 1C), with the levels being lowered to those observed in BALB/c mice with chronic toxoplasmosis treated with guanabenz (16, 17). These findings show that coupling guanabenz with a drug that potently targets tachyzoites is more efficacious than guanabenz alone in reducing cyst counts in latently infected mice.

Coadministration of pyrimethamine or clindamycin with guanabenz in BALB/c mice. In contrast to C57BL/6 mice, which are more susceptible to *Toxoplasma* infection than BALB/c mice, guanabenz alone is sufficient to significantly lower the brain cyst counts in latently infected BALB/c mice (16, 17). Given the synergy of the combination treatment in C57BL/6 mice, we sought to apply it to the BALB/c mouse model of infection in hopes that it would completely eliminate the cysts in these more resistant mice. Moreover, in addition to pyrimethamine, we tested a combination of guanabenz with another clinically used antiparasitic effective against tachyzoites: clindamycin (Fig. 2A) (21–23). Unfortunately, the addition of neither pyrimethamine nor clindamycin with guanabenz showed a further reduction in brain cyst counts compared with those achieved with guanabenz alone after 3 weeks of treatment (Fig. 2B). These findings indicate that a residual population of brain cysts (~20% to 30%) remains in BALB/c mice when guanabenz is administered alone or in combination with an antitachyzoite agent.

Effects of guanabenz combined with an ELQ against *Toxoplasma*. The current therapeutics against toxoplasmosis fail to eradicate the infection because they do not have significant activity against tissue cysts. We hypothesized that combination therapies composed of drugs with cyst-reducing capabilities may have synergistic effects. Guanabenz and endochin-like quinolones (ELQs) exhibit activity against tachyzoites and reduce brain cyst counts 70% to 80% through different mechanisms of action (12, 14). To address our hypothesis, we first examined the effects of guanabenz, ELQ-316, or both on type II Prugniad (Pru) strain tachyzoites in culture. A plaque assay showed an

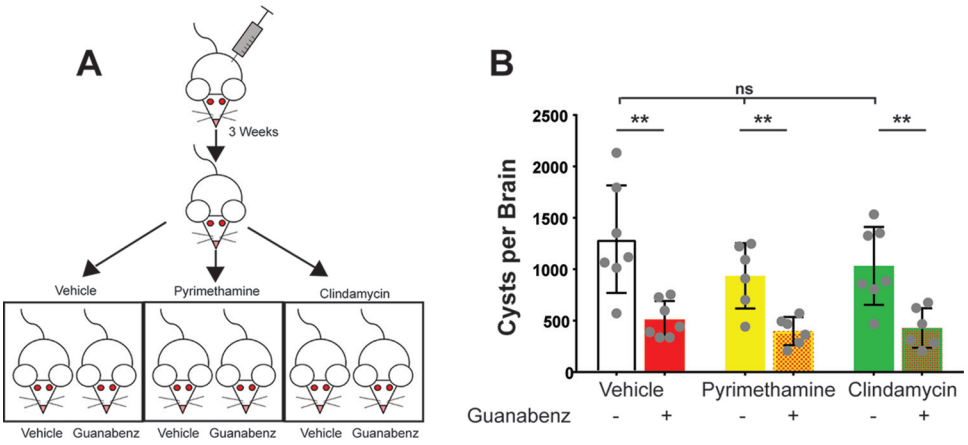

FIG 2 Effect of pyrimethamine or clindamycin with guanabenz on BALB/c brain cyst burden. (A) Six-week old, female BALB/c mice were infected intraperitoneally with *Toxoplasma* (gray syringe) and allowed to progress to chronic infection for 3 weeks. The latently infected mice were then randomized into six different treatment groups ($n = 7$). (B) Following 3 weeks of the indicated drug treatment, the brain cyst burden was quantified in a blind manner. *P* values were determined by one-way ANOVA ($P < 0.0001$), followed by an unpaired Student *t* test. ns, not significant; **, $P < 0.01$.

additive effect for guanabenz and ELQ-316, with minimal plaques being seen at low doses and no visible plaques being seen at the high doses (Fig. 3A). Synergistic activity against tachyzoite replication was also observed in a parasite doubling assay (Fig. 3B). The same synergistic activity against tachyzoites was observed for another type II parasite strain, ME49 (data not shown).

When guanabenz was added to tissue cysts generated *in vitro*, the bradyzoites took on an anomalous morphology. Bradyzoites within the guanabenz-treated cysts appeared to shrivel up, resulting in large empty spaces within the cyst (Fig. 4, black arrows). Treatment of tissue cysts with ELQ-316 was also damaging, resulting in cysts

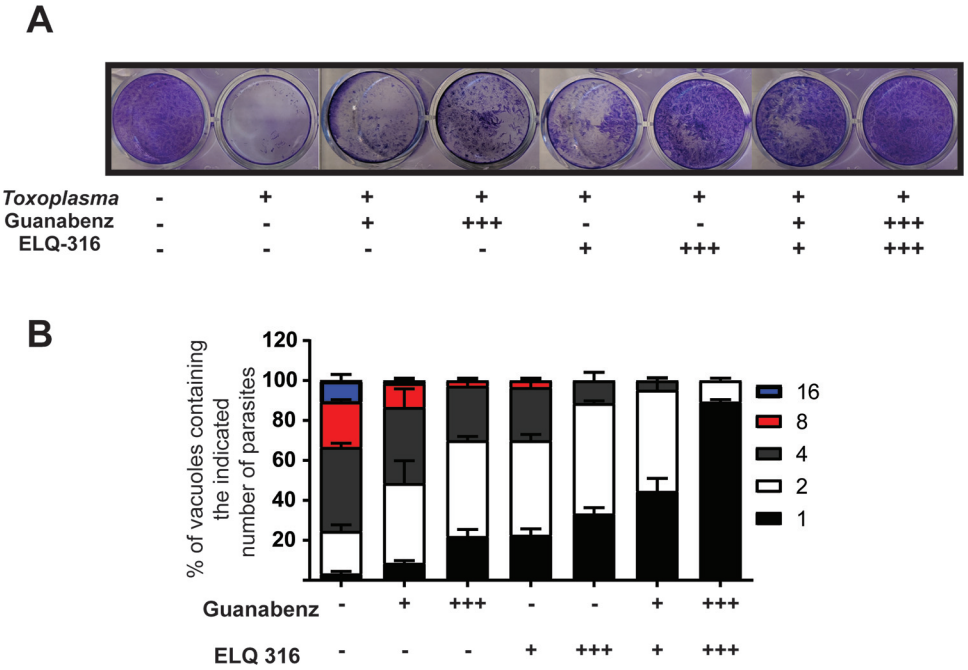

FIG 3 Additive effects of guanabenz and ELQ-316 *in vitro*. (A) Plaque assay of an HFF monolayer with or without *Toxoplasma* and the indicated drug concentrations. (B) Doubling assay with Pru tachyzoites after 36 h of drug treatment, performed with three biological replicates, each done in technical triplicate. +, 50% inhibitory concentration of the drug; +++, 3× 50% inhibitory concentration of the drug.

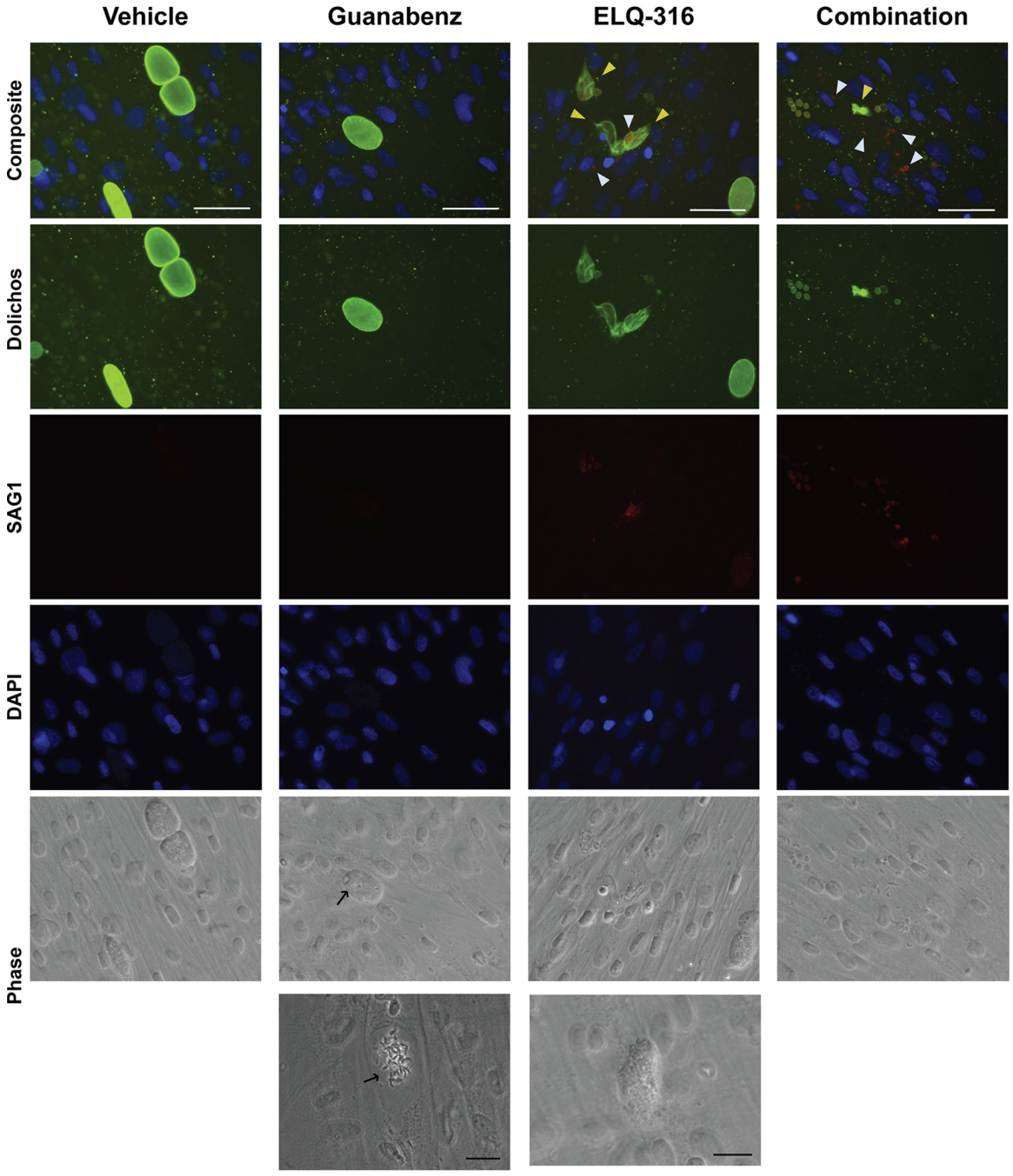

FIG 4 Guanabenz and ELQ-316 cause an abnormal cyst morphology. Bradyzoite cysts were generated by pH stress for 5 days *in vitro* before being drug treated for 4 days. DAPI, 4′,6-diamidino-2-phenylindole. Black arrows, swollen cyst structure with disorganized parasites inside; yellow arrowheads, shrunken cyst wall; gray arrowheads, SAG1-positive parasites. White bars = 50 μm; black bars = 20 μm.

that appeared shrunken and collapsed (Fig. 4, yellow arrowheads). In addition to using *Dolichos* lectin to stain the cyst walls, we stained these cultures for the tachyzoite surface antigen SAG1. No significant staining with SAG1 was evident in tissue cyst cultures treated with guanabenz alone. However, in the ELQ-316-treated tissue cysts, an increased number of SAG1-positive parasites was observed (Fig. 4, gray arrowheads). When the two drugs were administered together, SAG1-positive parasites were evident both within and outside of the tissue cysts, suggesting that these treated bradyzoites may have been converting to tachyzoites before dying.

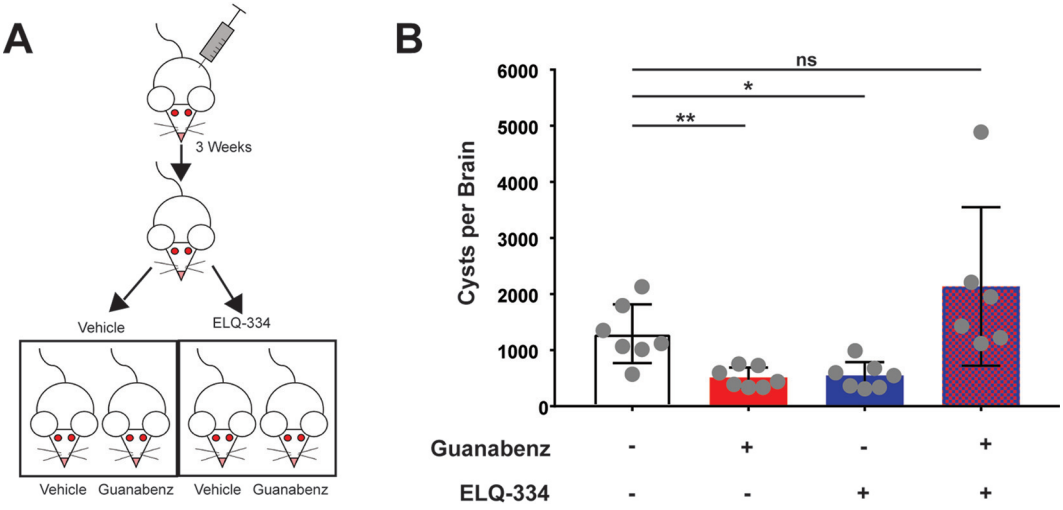

FIG 5 ELQ-334 does not have an additive effect with guanabenz in BALB/c mice. (A) Six-week old, female BALB/c mice were infected intraperitoneally with *Toxoplasma* (gray syringe) and allowed to progress to chronic infection for 3 weeks. The latently infected mice were then randomized into four different treatment groups ($n = 7$). (B) Following 3 weeks of the indicated drug treatment, the brain cyst burden was quantified in a blind manner. *P* values were determined by one-way ANOVA ($P = 0.0015$), followed by an unpaired Student *t* test. ns, not significant; *, $P < 0.05$; **, $P < 0.01$.

Given the promising results *in vitro*, we proceeded to test the efficacy of guanabenz and ELQ combination therapy in chronically infected BALB/c mice (Fig. 5A). ELQ-334 is a prodrug form of ELQ-316 designed to have increased bioavailability and stability (24). BALB/c mice with latent toxoplasmosis were treated with guanabenz, ELQ-334, or both for 3 weeks. As expected, both guanabenz and ELQ-334 monotherapy reduced the number of brain cysts; surprisingly, the combination therapy had no effect on cyst counts (Fig. 5B). Rather than being synergistic, these drugs nullify one another's ability to reduce the number of brain cysts *in vivo*.

Guanabenz-ELQ-pyrimethamine does not outperform monotherapy. Figure 4 reveals a possible explanation for the failed guanabenz-ELQ combination therapy: the SAG1-positive parasites observed in drug-treated cyst cultures could be a source of tachyzoites that reseed the brain with cysts. To test this possibility, we added pyrimethamine to the guanabenz-ELQ therapy (Fig. 6A).

The experimental setup accounted for all possible combinations of the three drugs in an attempt to better understand the mechanism for the failure of the guanabenz-ELQ treatment. We again observed no reduction in brain cyst counts when guanabenz and ELQ-334 were coadministered but were able to reestablish a statistically significant reduction in the cyst burden when all three drugs were given (Fig. 6B). However, the triple therapy did not significantly lower the cyst burden below that achieved with guanabenz or ELQ monotherapy.

Since C57BL/6 mice are more sensitive to tachyzoites than BALB/c mice, we sought to confirm the improved efficacy of the triple therapy in this mouse strain (18, 19). We hypothesized that the triple therapy would display better efficacy in C57BL/6 mice than in BALB/c mice, based on the improvement seen when pyrimethamine was combined with guanabenz (Fig. 1C). A schematic of the experimental design is shown in Fig. 7A.

Given that the combination of guanabenz and ELQ-334 failed to reduce the brain cyst counts in BALB/c mice (Fig. 5B), we were not surprised to see reduced survival in some of the latently infected C57BL/6 mice treated with this combination, though the difference did not reach statistical significance (Fig. 7B). However, the inclusion of pyrimethamine did not improve survival rates, which suggests continued failure of the triple therapy in controlling infection (Fig. 7B). The brain cyst burdens in the survivors of the guanabenz-ELQ group and the guanabenz-ELQ-pyrimethamine triple-therapy group were significantly reduced, but the loss of almost half of the group precluded practical application without further optimization (Fig. 7C). Interestingly, this experi-

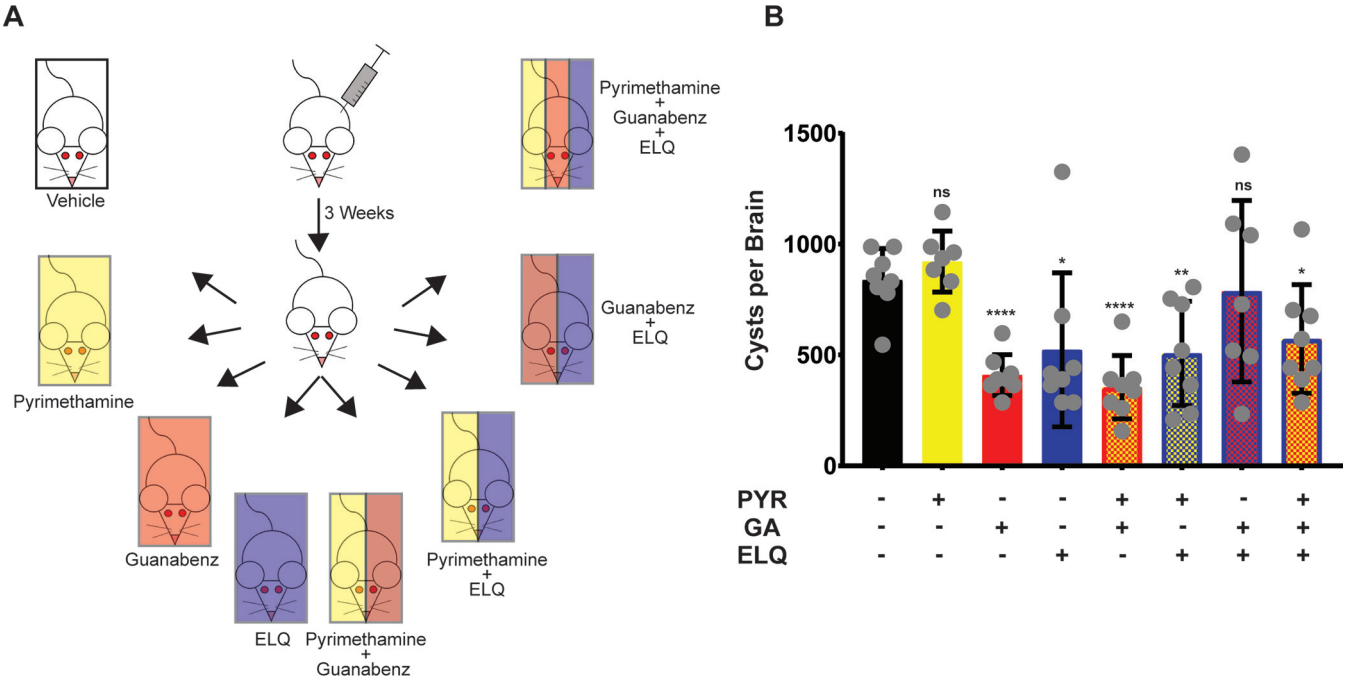

FIG 6 Triple-combination therapy in BALB/c mice. (A) Six-week old, female BALB/c mice were infected intraperitoneally with *Toxoplasma* (gray syringe), allowed to progress to chronic infection for 3 weeks, and then randomized into eight different treatment groups. (B) Following 3 weeks of the indicated drug treatment, the brain cyst burden was quantified in a blind manner. *P* values were determined by one-way ANOVA ($P < 0.0001$), followed by an unpaired Student *t* test. The statistics shown are for comparison to the vehicle control. ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$.

ment revealed that ELQ-334 combined with pyrimethamine impressively reduces brain cyst counts 95%, to nearly undetectable levels, in C57BL/6 mice. This finding suggests that further optimization with ELQs paired with pyrimethamine or perhaps another antitachyzoite agent may be a promising avenue toward the eradication of toxoplasmosis.

DISCUSSION

We recently showed that the efficacy of guanabenz against latent toxoplasmosis varies in different strains of mice. Guanabenz significantly reduces brain cyst counts in BALB/c mice, but in C57BL/6 mice, guanabenz treatment was lethal for half the group and resulted in an increase in the cyst burden among the survivors (17). Here, we endeavored to prevent the loss of C57BL/6 mice through the coadministration of a second drug. The inclusion of pyrimethamine prevented the guanabenz-induced death; moreover, the C57BL/6 mice receiving the guanabenz-pyrimethamine cotreatment showed a significant reduction in their brain cyst burdens (Fig. 1C). This success encouraged us to pursue additional combination therapies with guanabenz in hopes of completely eliminating parasites in these mice.

Combination therapy is currently the standard of care against acute toxoplasmosis (7). The first-line drug combination is pyrimethamine and sulfadiazine, which target two independent steps of the folic acid synthesis pathway, depriving the parasites of purines. While they are effective against acute toxoplasmosis, allergies or adverse effects in response to sulfa drugs force a switch to another therapeutic (25). The second-line drug combination is pyrimethamine and clindamycin, the latter of which targets translation in the parasite-specific plastid-like organelle called the apicoplast (26).

Since pyrimethamine and clindamycin have different targets and are clinically available, we tested their efficacy with guanabenz against latent toxoplasmosis. Our results showed no improvement in brain cyst burden reduction in BALB/c mice. This was not surprising, given that BALB/c mice, unlike C57BL/6 mice, showed no signs of

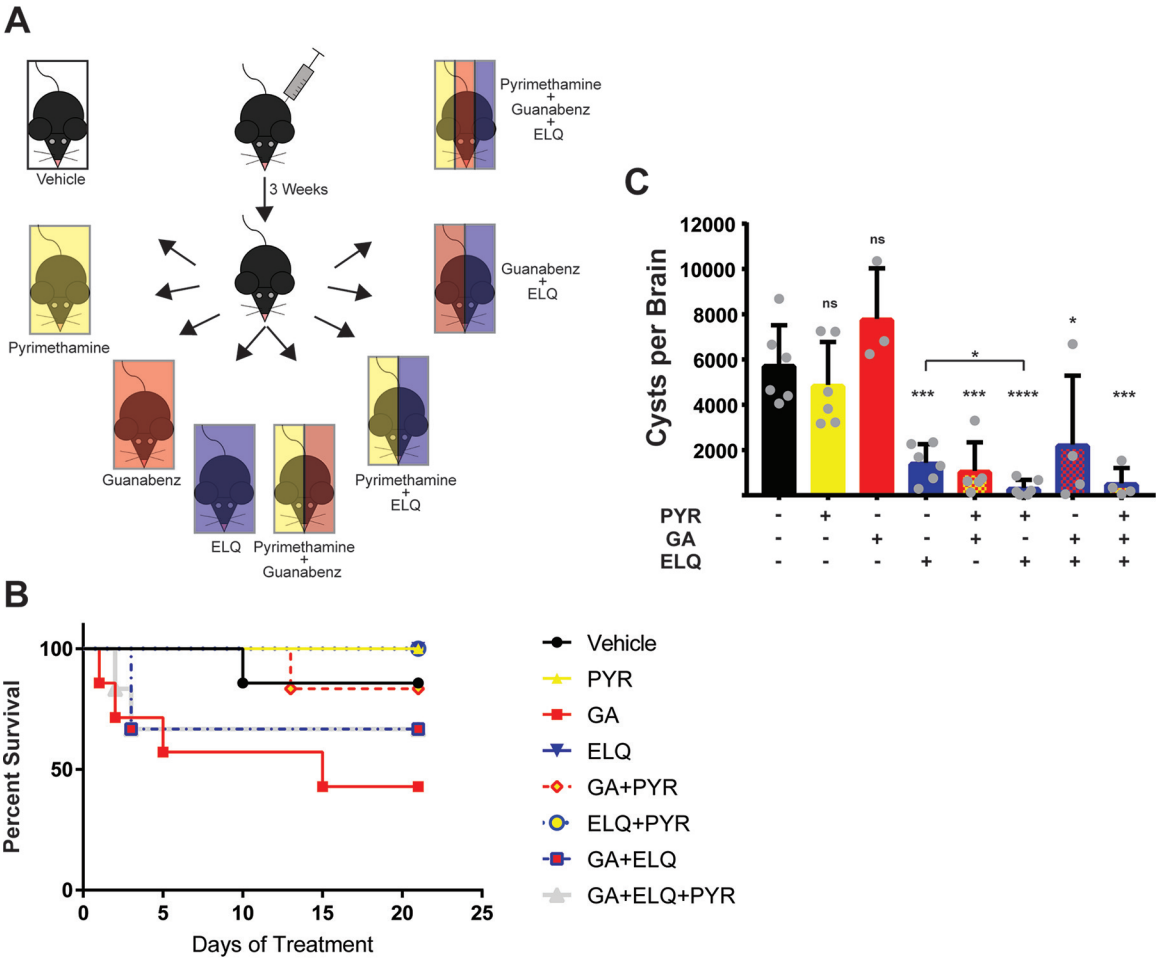

FIG 7 Triple-combination therapy in C57BL/6 mice. (A) Six-week-old, female C57BL/6 mice were infected intraperitoneally with *Toxoplasma* (gray syringe), allowed to progress to chronic infection for 3 weeks, and then randomized into eight different treatment groups ($n = 6$). (B) The latently infected mice were treated with the indicated drugs for 3 weeks, and survival was tracked over that time. The difference was not statistically significant by the log-rank test. (C) Following 3 weeks of treatment, the brain cyst burden was quantified in a blind manner. *P* values were determined by one-way ANOVA ($P < 0.0001$), followed by an unpaired Student *t* test. Statistics above the bar are for comparison to the vehicle control unless indicated otherwise. ns, not significant; *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$.

reactivated toxoplasmosis or any indication that there were tachyzoites present during guanabenz treatment. Since both pyrimethamine and clindamycin rely on actively replicating parasites to be effective, the lack of tachyzoites in BALB/c mice likely explains the ineffectiveness of this combination therapy. It is possible that the residual population of cysts resides in an area of brain that is inaccessible to the drugs.

We also addressed the idea that the coadministration of another cyst-reducing drug with guanabenz would have synergistic effects, potentially lowering the cyst counts to a point below the threshold of detection. Interestingly, none of the compounds studied to date have been able to eradicate brain cysts completely when they are used as a monotherapy. Ours is the first study to examine a combination of two cyst-reducing drugs.

ELQs, which have a mechanism of action different from that of guanabenz, show a 76% to 88% reduction of brain cysts in CBA/J mice (14). *In vitro* studies showed a synergistic effect of guanabenz and ELQ-316 against tachyzoites, as evidenced in two independent parasite growth assays (Fig. 3). Treatment of *in vitro*-generated bradyzoite cysts resulted in gross morphological changes that were amplified when the drugs were used in combination. Cysts treated with guanabenz were swollen, with empty spaces and deformed bradyzoites being visible within the cyst lumen, as previously reported (16). Cysts treated with ELQ-316 showed a shrunken morphology, resembling

a raisin, with some parasites expressing SAG1. When the drugs were combined, there was an abundance of small, sometimes shrunken cysts that expressed SAG1, in addition to larger shrunken cysts and a number of SAG1-positive parasites outside of cyst walls.

When we coadministered guanabenz and ELQ-334 (the prodrug of ELQ-316) *in vivo* for the treatment of latent toxoplasmosis in BALB/c mice, we did not observe the additive effect that we anticipated; in fact, we no longer observed the decrease in brain cyst burden normally seen in response to monotherapy with either drug. In other words, the drugs appeared to be antagonistic *in vivo* through a mechanism that remains to be determined. The presence of SAG1-positive parasites seen *in vitro* with ELQ treatment could potentially explain its lack of effect when combined with guanabenz *in vivo*. We tested this possibility using a triple-combination treatment by adding pyrimethamine, which we had previously shown to be efficacious in a system in which tachyzoites are likely present as well. The triple-drug combination was effective in significantly reducing the cyst burden in BALB/c mice, but it did not lower it as much as the monotherapies did.

Since we previously established that guanabenz works differently against latent toxoplasmosis in different mouse strains, we also performed combination drug treatments in C57BL/6 mice (17). It was unsurprising that guanabenz with ELQ-334 caused several of the mice to succumb to toxoplasmosis, since this combination failed to reduce the cyst burden in BALB/c mice. The addition of pyrimethamine did not help prevent the lethality seen in some of the mice given guanabenz-ELQ; however, the surviving mice displayed reduced cyst counts. Interestingly, the coadministration of pyrimethamine and ELQ-334 resulted in an impressive reduction in the brain cyst burden in the C57BL/6 mice down toward undetectable limits.

In conclusion, while the addition of pyrimethamine to guanabenz treatment was effective in reducing the cyst burden and preventing death in C57BL/6 mice, it was not more efficacious than guanabenz alone in BALB/c mice. While the combination of guanabenz with ELQ-334 showed additive effects *in vitro*, it was not effective in either mouse model *in vivo*. However, the combination of ELQ-334 with pyrimethamine proved more effective than ELQ-334 alone, emerging as a promising combination that could be further optimized in the future.

MATERIALS AND METHODS

Parasite strains and culture. *Toxoplasma gondii* parasites (type II Prugniad [Pru] strain) were collected as tissue cysts from the brains of chronically infected BALB/c mice and used to infect human foreskin fibroblasts (HFFs). The infected cultures were maintained in Dulbecco's medium supplemented with 1% heat-inactivated fetal bovine serum (FBS) in a humidified incubator at 37°C with 5% CO_2. To ensure their developmental capacity, tachyzoites were maintained in culture for no more than 15 passages. The parasites were tested for mycoplasma using PCR, as previously described (27), prior to their use in mice.

Mouse strains and infection. The mice used in this study were housed in Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved facilities at either the Indiana University School of Medicine Laboratory Animal Research Center (LARC) or the IUPUI Science Animal Research Center (SARC). The Institutional Animal Care and Use Committee (IACUC) at the Indiana University School of Medicine approved the use of all animals and procedures (IACUC protocol number 11376).

BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME) at 5 weeks of age. The mice were allowed to acclimate for 1 week before being intraperitoneally (i.p.) infected with 10^4 Pru tachyzoites suspended in 100 μl of autoclaved, filter-sterilized phosphate-buffered saline (PBS). C57BL/6 mice, also purchased from The Jackson Laboratory at 5 weeks of age, were infected with 10^3 Pru tachyzoites. The mice were routinely observed multiple times a day throughout the course of the acute infection. After 21 days postinfection, the mice were randomized into treatment groups. At the conclusion of the study, blood samples were extracted by cardiac puncture from all mice to confirm parasite infection through serological analysis of the collected serum to identify parasite-specific antibodies using a dot blot containing parasite lysate.

Drug sources and administration. Stock solutions were made at the beginning of the experiment and aliquoted in 1.5-ml Eppendorf tubes, and the tubes were stored at 4°C until needed. Guanabenz (Sigma) was delivered i.p. at 5 mg/kg of body weight daily in sterile saline. Pyrimethamine (MP Biomedicals) and clindamycin (Sigma) were dosed i.p. at 20 mg/kg daily in sterile saline. ELQ-316 and ELQ-334 were synthesized as described in the accompanying article (20), identified by proton nuclear magnetic resonance (^{1}H NMR), and determined to be >95% pure by reversed-phase high-performance liquid chromatography (HPLC). ELQ-334 was administered by oral gavage, using a flexible plastic gavage needle, at 10 mg/kg daily in corn oil. The treatment time for each drug was 3 weeks.

Brain cyst quantification. The brains were extracted upon euthanasia, and the cyst burden was quantified as previously described (16). Briefly, the dissected tissue was homogenized in 650 μl of sterile PBS using a mortar and pestle. A 250-μl aliquot of homogenate was fixed using 3% methanol-free formaldehyde for 20 min. The homogenate was blocked using 3% bovine serum albumin (BSA) in 0.2% Triton X-100 before staining with 1:250 rhodamine-conjugated *Dolichos biflorus* lectin (Vector Laboratories Inc.) to visualize the cyst wall. Five-microliter aliquots of stained homogenate were placed on a coverslip, and the coverslip was sealed before imaging. The stained samples were deidentified before the cysts were counted under a $\times$20 magnification. The count was then extrapolated to estimate the cyst burden for the entire brain.

***In vitro* parasite viability assays.** For the plaque assay, 12-well plates containing confluent HFF monolayers were infected with 10^4 Pru tachyzoites, and the tachyzoites were allowed to invade the HFFs for 2 h. The plates were then washed with warm sterile PBS twice before fresh medium supplemented with the indicated drug was added. The plates were left undisturbed under normal culture conditions for 13 days. They were then fixed with 100% ice-cold methanol for 20 min and stained with crystal violet for 20 min. For parasite doubling assays, 12-well plates of HFFs were infected with 10^3 Pru tachyzoites, and the tachyzoites were allowed to invade the HFFs for 2 h. The plates were then washed with warm sterile PBS twice before fresh medium supplemented with the indicated drug was added. The plates were left undisturbed under normal culture conditions for 36 h. They were then fixed with 100% ice-cold methanol for 20 min and washed with PBS. The number of parasites within 50 randomly chosen vacuoles was counted.

***In vitro* parasite differentiation.** HFF monolayers were grown on coverslips in 12-well plates for 3 days until they were confluent. The parasites were allowed to invade the monolayer for 2 h under normal culture conditions, and then the infected monolayers were washed with warm sterile PBS 3 times to remove uninvaded parasites. We then applied RPMI without sodium bicarbonate (Sigma-Aldrich) medium, adjusted to pH 8.2 with NaOH. The plates were sealed in plastic bags and incubated at 37°C under ambient CO_2. The medium was replaced every other day to maintain an alkaline pH.

Statistics. Statistical analysis was performed using GraphPad Prism (version 7.03) software. For data sets comparing two groups, statistical significance was determined using Student's *t* test. If more than two groups were compared, the data sets were analyzed using one-way analysis of variance (ANOVA) to determine statistical significance before secondary analysis with Student's *t* test. Survival data were analyzed using a log-rank test. *P* values of $\leq$0.05 were considered statistically significant.

ACKNOWLEDGMENTS

This research was supported by grants from the National Institutes of Health (grants AI124723 and AI150616 to W.J.S.) and Career Development Award BX002440 and VA Merit Review Award BX004522 (to J.S.D.). J.M. was supported by PHS grant T32 AI060519 and a Joseph and Lucille Madri Family scholarship.

REFERENCES

1. Flegr J, Prandota J, Sovickova M, Israili ZH. 2014. Toxoplasmosis—a global threat. Correlation of latent toxoplasmosis with specific disease burden in a set of 88 countries. PLoS One 9:e90203. https://doi.org/10.1371/journal.pone.0090203.
2. Delgado Betancourt E, Hamid B, Fabian BT, Klotz C, Hartmann S, Seeber F. 2019. From entry to early dissemination—Toxoplasma gondii's initial encounter with its host. Front Cell Infect Microbiol 9:46. https://doi.org/10.3389/fcimb.2019.00046.
3. Porter SB, Sande MA. 1992. Toxoplasmosis of the central nervous system in the acquired immunodeficiency syndrome. N Engl J Med 327:1643–1648. https://doi.org/10.1056/NEJM199212033272306.
4. Wohlfert EA, Blader IJ, Wilson EH. 2017. Brains and brawn: Toxoplasma infections of the central nervous system and skeletal muscle. Trends Parasitol 33:519–531. https://doi.org/10.1016/j.pt.2017.04.001.
5. Mulanovich VE, Ahmed SI, Ozturk T, Khokhar FA, Kontoyiannis DP, de Lima M. 2011. Toxoplasmosis in allo-SCT patients: risk factors and outcomes at a transplantation center with a low incidence. Bone Marrow Transplant 46:273–277. https://doi.org/10.1038/bmt.2010.102.
6. Cabral CM, Tuladhar S, Dietrich HK, Nguyen E, MacDonald WR, Trivedi T, Devineni A, Koshy AA. 2016. Neurons are the primary target cell for the brain-tropic intracellular parasite Toxoplasma gondii. PLoS Pathog 12:e1005447. https://doi.org/10.1371/journal.ppat.1005447.
7. Dunay IR, Gajurel K, Dhakal R, Liesenfeld O, Montoya JG. 2018. Treatment of toxoplasmosis: historical perspective, animal models, and current clinical practice. Clin Microbiol Rev :31:e00057-17. https://doi.org/10.1128/CMR.00057-17.
8. Yan J, Huang B, Liu G, Wu B, Huang S, Zheng H, Shen J, Lun ZR, Wang Y, Lu F. 2013. Meta-analysis of prevention and treatment of toxoplasmic encephalitis in HIV-infected patients. Acta Trop 127:236–244. https://doi.org/10.1016/j.actatropica.2013.05.006.
9. Cohn JA, McMeeking A, Cohen W, Jacobs J, Holzman RS. 1989. Evaluation of the policy of empiric treatment of suspected Toxoplasma encephalitis in patients with the acquired immunodeficiency syndrome. Am J Med 86:521–527. https://doi.org/10.1016/0002-9343(89)90378-1.
10. Haverkos HW. 1987. Assessment of therapy for toxoplasma encephalitis. The TE Study Group. Am J Med 82:907–914. https://doi.org/10.1016/0002-9343(87)90151-3.
11. Vidadala RS, Rivas KL, Ojo KK, Hulverson MA, Zambriski JA, Bruzual I, Schultz TL, Huang W, Zhang Z, Scheele S, DeRocher AE, Choi R, Barrett LK, Siddaramaiah LK, Hol WG, Fan E, Merritt EA, Parsons M, Freiberg G, Marsh K, Kempf DJ, Carruthers VB, Isoherranen N, Doggett JS, Van Voorhis WC, Maly DJ. 2016. Development of an orally available and central nervous system (CNS) penetrant Toxoplasma gondii calcium-dependent protein kinase 1 (TgCDPK1) inhibitor with minimal human ether-a-go-go-related gene (hERG) activity for the treatment of toxoplasmosis. J Med Chem 59:6531–6546. https://doi.org/10.1021/acs.jmedchem.6b00760.
12. McConnell EV, Bruzual I, Pou S, Winter R, Dodean RA, Smilkstein MJ, Krollenbrock A, Nilsen A, Zakharov LN, Riscoe MK, Doggett JS. 2018. Targeted structure-activity analysis of endochin-like quinolones reveals potent Qi and Qo site inhibitors of Toxoplasma gondii and Plasmodium falciparum cytochrome bc1 and identifies ELQ-400 as a remarkably effective compound against acute experimental toxoplasmosis. ACS Infect Dis 4:1574–1584. https://doi.org/10.1021/acsinfecdis.8b00133.
13. Alday PH, Bruzual I, Nilsen A, Pou S, Winter R, Ben Mamoun C, Riscoe MK, Doggett JS. 2017. Genetic evidence for cytochrome b Qi site inhibition by 4(1H)-quinolone-3-diarylethers and antimycin in Toxoplasma gondii. Antimicrob Agents Chemother 61:e01866-16. https://doi.org/10.1128/AAC.01866-16.
14. Doggett JS, Nilsen A, Forquer I, Wegmann KW, Jones-Brando L, Yolken

RH, Bordon C, Charman SA, Katneni K, Schultz T, Burrows JN, Hinrichs DJ, Meunier B, Carruthers VB, Riscoe MK. 2012. Endochin-like quinolones are highly efficacious against acute and latent experimental toxoplasmosis. Proc Natl Acad Sci U S A 109:15936–15941. https://doi.org/10.1073/pnas.1208069109.
15. Konrad C, Queener SF, Wek RC, Sullivan WJ, Jr. 2013. Inhibitors of eIF2alpha dephosphorylation slow replication and stabilize latency in Toxoplasma gondii. Antimicrob Agents Chemother 57:1815–1822. https://doi.org/10.1128/AAC.01899-12.
16. Benmerzouga I, Checkley LA, Ferdig MT, Arrizabalaga G, Wek RC, Sullivan WJ, Jr. 2015. Guanabenz repurposed as an antiparasitic with activity against acute and latent toxoplasmosis. Antimicrob Agents Chemother 59:6939–6945. https://doi.org/10.1128/AAC.01683-15.
17. Martynowicz J, Augusto L, Wek RC, Boehm SL, II, Sullivan WJ, Jr. 2019. Guanabenz reverses a key behavioral change caused by latent toxoplasmosis in mice by reducing neuroinflammation. mBio 10:e00381-19. https://doi.org/10.1128/mBio.00381-19.
18. Suzuki Y, Orellana MA, Schreiber RD, Remington JS. 1988. Interferon-gamma: the major mediator of resistance against Toxoplasma gondii. Science 240:516–518. https://doi.org/10.1126/science.3128869.
19. Suzuki Y, Sher A, Yap G, Park D, Neyer LE, Liesenfeld O, Fort M, Kang H, Gufwoli E. 2000. IL-10 is required for prevention of necrosis in the small intestine and mortality in both genetically resistant BALB/c and susceptible C57BL/6 mice following peroral infection with Toxoplasma gondii. J Immunol 164:5375–5382. https://doi.org/10.4049/jimmunol.164.10.5375.
20. Doggett JS, Schultz T, Miller AJ, Bruzual I, Pou S, Winter R, Dodean R, Zakharov LN, Nilsen A, Riscoe MK, Carruthers VB. 2020. Orally bioavailable endochin-like quinolone carbonate ester prodrug reduces *Toxoplasma gondii* brain cysts. Antimicrob Agents Chemother 64:e00535-20. https://doi.org/10.1128/AAC.00535-20.
21. Djurkovic-Djakovic O, Nikolic T, Robert-Gangneux F, Bobic B, Nikolic A. 1999. Synergistic effect of clindamycin and atovaquone in acute murine toxoplasmosis. Antimicrob Agents Chemother 43:2240–2244. https://doi.org/10.1128/AAC.43.9.2240.
22. Araujo FG, Remington JS. 1974. Effect of clindamycin on acute and chronic toxoplasmosis in mice. Antimicrob Agents Chemother 5:647–651. https://doi.org/10.1128/aac.5.6.647.
23. Hofflin JM, Remington JS. 1987. Clindamycin in a murine model of toxoplasmic encephalitis. Antimicrob Agents Chemother 31:492–496. https://doi.org/10.1128/aac.31.4.492.
24. Lawres LA, Garg A, Kumar V, Bruzual I, Forquer IP, Renard I, Virji AZ, Boulard P, Rodriguez EX, Allen AJ, Pou S, Wegmann KW, Winter RW, Nilsen A, Mao J, Preston DA, Belperron AA, Bockenstedt LK, Hinrichs DJ, Riscoe MK, Doggett JS, Ben Mamoun C. 2016. Radical cure of experimental babesiosis in immunodeficient mice using a combination of an endochin-like quinolone and atovaquone. J Exp Med 213:1307–1318. https://doi.org/10.1084/jem.20151519.
25. Hernandez AV, Thota P, Pellegrino D, Pasupuleti V, Benites-Zapata VA, Deshpande A, Penalva de Oliveira AC, Vidal JE. 2017. A systematic review and meta-analysis of the relative efficacy and safety of treatment regimens for HIV-associated cerebral toxoplasmosis: is trimethoprim-sulfamethoxazole a real option? HIV Med 18:115–124. https://doi.org/10.1111/hiv.12402.
26. Camps M, Arrizabalaga G, Boothroyd J. 2002. An rRNA mutation identifies the apicoplast as the target for clindamycin in Toxoplasma gondii. Mol Microbiol 43:1309–1318. https://doi.org/10.1046/j.1365-2958.2002.02825.x.
27. Hopert A, Uphoff CC, Wirth M, Hauser H, Drexler HG. 1993. Specificity and sensitivity of polymerase chain reaction (PCR) in comparison with other methods for the detection of mycoplasma contamination in cell lines. J Immunol Methods 164:91–100. https://doi.org/10.1016/0022-1759(93)90279-g.