Guanabenz Repurposed as an Antiparasitic with Activity against Acute and Latent Toxoplasmosis

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Toxoplasma gondii is a protozoan parasite that persists as a chronic infection. *Toxoplasma* evades immunity by forming tissue cysts, which reactivate to cause life-threatening disease during immune suppression. There is an urgent need to identify drugs capable of targeting these latent tissue cysts, which tend to form in the brain. We previously showed that translational control is critical during infections with both replicative and latent forms of *Toxoplasma*. Here we report that guanabenz, an FDA-approved drug that interferes with translational control, has antiparasitic activity against replicative stages of *Toxoplasma* and the related apicomplexan parasite *Plasmodium falciparum* (a malaria agent). We also found that inhibition of translational control interfered with tissue cyst biology *in vitro*. *Toxoplasma* bradyzoites present in these abnormal cysts were diminished and misconfigured, surrounded by empty space not seen in normal cysts. These findings prompted analysis of the efficacy of guanabenz *in vivo* by using established mouse models of acute and chronic toxoplasmosis. In addition to protecting mice from lethal doses of *Toxoplasma*, guanabenz has a remarkable ability to reduce the number of brain cysts in chronically infected mice. Our findings suggest that guanabenz can be repurposed into an effective antiparasitic with a unique ability to reduce tissue cysts in the brain.

According to the Centers for Disease Control and Prevention, over 60 million people in the United States may be infected with the intracellular protozoan parasite *Toxoplasma gondii*. In other regions of the world, seroprevalence has been estimated to be as high as 80% (1). This apicomplexan parasite has obtained such an extensive distribution among humans and other animals through its multiple routes of transmission (2). *Toxoplasma* can be spread by inhalation or ingestion of oocysts expelled by infected felines, the definitive host of the parasite. Upon infection, the replicative phase of the parasite (tachyzoite) converts into a more quiescent phase (bradyzoite) as intracellular tissue cysts, which can facilitate further spread of the parasite if ingested by a predator. Unfortunately, *Toxoplasma* is also capable of crossing the placental barrier, making mother-to-child infection yet another mode of transmission for the parasite. Between 400 and 4,000 cases of congenital toxoplasmosis occur in the United States each year, which can result in severe birth defects that include vision impairment and neurological effects (3).

Most infections with *Toxoplasma* are asymptomatic due to the host immune response, quickly prompting transformation of the parasite from the tachyzoite stage to the latent bradyzoite stage. The bradyzoites form tissue cysts, which constitute a chronic infection that the host is thought to retain for life. Severe health risks arise when the immunity of an infected individual becomes impaired, which can occur in AIDS patients, triggering conversion of the bradyzoites into rapidly replicating tachyzoites (2). Reactivation of infection results in an episode of acute toxoplasmosis that can be life-threatening and necessitates treatment with drugs, such as the antifolate regimen of pyrimethamine and sulfadiazine (4). Currently, there is no approved therapy that eradicates the tissue cysts responsible for chronic infection, and the untoward side effects of antifolates severely limit their utility in patients. In short, new therapies that can target both the tachyzoite and bradyzoite stages are urgently needed.

We previously suggested that translational control through phosphorylation of parasite eukaryotic initiation factor 2α (eIF2α) is important for the development and maintenance of latent forms of *Toxoplasma* and another apicomplexan parasite, *Plasmodium* (a malaria agent) (5, 6). eIF2 is a well-conserved factor that delivers initiator tRNA to ribosomes during protein synthesis (7). Upon cellular stress, the alpha subunit of eIF2 is phosphorylated, curbing general protein synthesis coincident with preferential translation of a subset of mRNA transcripts that are involved in stress amelioration (7, 8). Our previous studies of *Toxoplasma* established that TgIF2α phosphorylation is critical during the lytic cycle in tachyzoites and also occurs in response to stresses that induce conversion of tachyzoites to bradyzoites *in vitro* (9–11). Since translational control has roles in both of these clinically relevant parasite stages, TgIF2α phosphorylation is a process that shows great promise as a novel antiparasitic drug target. To address this idea, we showed that inhibitors of eIF2α dephosphorylation, namely, guanabenz and salubrinal, have potent activity against *Toxoplasma in vitro* (12). We further demonstrated that these drugs could act directly on the parasite to maintain TgIF2α phosphorylation and that their ability to kill *Toxoplasma* did not involve host eIF2α (10, 12). The anti-*Toxoplasma* activity of guanabenz is also promising because this drug is already FDA approved, with a proven safety record that goes back

30 years ([13]). Importantly, guanabenz also crosses the blood-brain barrier ([14]), which is an important criterion for drug candidates, since bradyzoite tissue cysts have a propensity to form in the brain.

In the present study, we determined the activity of guanabenz against additional *Toxoplasma* strains as well as the fellow apicomplexan parasite *Plasmodium falciparum in vitro*, noting that guanabenz exhibits a unique ability to disrupt tissue cyst physiology. We further addressed the efficacy of guanabenz against both acute and chronic stages of infection by using established mouse models of toxoplasmosis. Consistent with the *in vitro* data, guanabenz not only protects mice from acute toxoplasmosis but also reduces the number of brain cysts in chronically infected animals. These results suggest that guanabenz may rapidly be repurposed as a novel antiparasitic agent that can dually target replicative and latent stages of the parasite.

MATERIALS AND METHODS

Chemical reagents. Guanabenz acetate was purchased from Sigma-Aldrich (G110) and dissolved in saline at a concentration of 2.5 mg/ml for storage at $-20°C$. Salubrinal was synthesized in collaboration with the Department of Chemistry and Chemical Biology, Indiana University–Purdue University at Indianapolis, with the assigned standard nomenclature IUSC-12447-000-A, and was stored at $-20°C$. Salubrinal was dissolved to 10 mg/ml in dimethyl sulfoxide (DMSO) and was made fresh for each use.

Parasite culture and replication assays. *Toxoplasma* parasites (strains ME49 and Prugniaud [Pru] BSG-4) ([15]) were maintained in human foreskin fibroblast (HFF) monolayers in Dulbecco's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Uninfected and infected HFF cells were maintained in a humidified incubator at 37°C with 5% CO_2.

To monitor *Toxoplasma* replication, a standard parasite counting assay was used ([16]). Briefly, 10^4 Pru parasites were allowed to infect a confluent HFF monolayer. At 4 h postinfection, extracellular parasites were removed and the medium was replaced with medium containing the indicated concentration of drug or vehicle. At 32 h postinfection, parasites were fixed with 1 ml ice-cold methanol for 10 min and then stained with Diff-Quick (Siemens) for 1 min to visualize parasites by light microscopy ([17]). The number of tachyzoites in each of 50 randomly chosen vacuoles was recorded.

P. falciparum strains HB3 (Honduras) and Dd2 (Indochina) were maintained in O^+ human red blood cells (Biochemed, Winchester, VA) and RPMI 1640 medium (Gibco) supplemented with 0.5% Albumax II (Gibco), 0.25% sodium bicarbonate (Corning), and 0.01 mg/ml gentamicin (Gibco) under an atmosphere of 90% nitrogen, 5% O_2, and 5% CO_2. Cultures underwent at least two life cycles prior to initiation of assays to ensure that normal growth was established. Dose-response curves were generated using a hypoxanthine incorporation assay ([18]). Briefly, parasite cultures for assays were maintained as asynchronous cultures and required to reach a parasitemia of no less than 1%, with 70% of parasites in the early ring stage. Sample parasitemia and hematocrit were then adjusted to 0.2% and 2%, respectively, and the samples were added to test plates containing 2-fold dilutions of guanabenz. Parasites were exposed to drug dilutions for 48 h. [^{3}H]hypoxanthine (PerkinElmer) was added to the plates and incubated for an additional 20 h before freezing at $-80°C$ for >24 h. Plate contents were harvested and counted on a Trilux beta counter. The dose-response curves show means for three independent biological replicates.

***In vitro* bradyzoite differentiation assays.** *Toxoplasma* tachyzoites were converted into bradyzoite tissue cysts *in vitro* by using previously described methods ([19]), with minor modifications. Approximately 0.5×10^6 parasites were allowed to infect 12- to 14-day-old HFF monolayers cultured in T-25 flasks under the normal conditions described above.

Four hours later, infected cells were washed to remove parasites that had not invaded host cells, and an alkaline medium was applied (pH 8.1). Flasks were kept in a humidified incubator at 37°C with ambient CO_2, and the alkaline medium was replaced every other day. To visualize cyst walls, flasks were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and stained with rhodamine-conjugated *Dolichos biflorus* agglutinin (Vector Laboratories).

***In vivo* assays.** Acute and chronic toxoplasmosis was studied *in vivo* by using female BALB/c mice of 4 to 6 weeks of age (Harlan Laboratories) that were maintained at the Indiana University School of Medicine according to institutional and IACUC guidelines. To study acute infection, mice were injected intraperitoneally (i.p.) with a lethal dose of tachyzoites (10^4 ME49 tachyzoites or 10^6 Pru tachyzoites). The indicated dose of guanabenz or saline vehicle was injected i.p. every 2 days, starting at 24 h postinfection. Mice were monitored at least twice a day, and the time to death was recorded. To establish chronic infection, mice were inoculated with a nonlethal dose of 10^5 Pru parasites. On day 25 postinfection, chronically infected mice were randomized and administered guanabenz or saline vehicle (i.p.) for 19 days, a treatment time based on previous studies (e.g., see reference [20]). For each mouse experiment, a portion of the parasite inoculum was used in a standard plaque assay; the plaque-forming efficiencies verified that there were no significant fitness differences between the parasites in the inoculums.

To determine cyst burdens, brains were homogenized in PBS, and a portion of each was analyzed to enumerate cysts or parasite burdens as described previously ([21]). For cyst counts, brain homogenates were fixed with 3% methanol-free formaldehyde (Thermo Scientific) for 20 min and blocked in 0.2% Triton X-100 with 3.0% bovine serum albumin (BSA) for 1 h. Cysts were visualized by staining with rhodamine-conjugated *Dolichos biflorus* lectin. For measurements of parasite burden, total genomic DNAs were isolated from brain homogenates by using DNeasy blood and tissue kits (Qiagen), and the *Toxoplasma* B1 gene was measured by quantitative PCR (qPCR) with the following primers: sense, 5′-GGAGGACT GGCAACCTGGTGTCG; and antisense, 5′-TTGTTTCACCCGGACCGT TTAGCAG ([22]). The number of parasites was deduced from a standard curve prepared in parallel.

RESULTS

Guanabenz inhibits replicative stages of *Toxoplasma* and *Plasmodium in vitro*. We previously showed that guanabenz inhibits *in vitro* replication of the type I RH and type II ME49 strains of *Toxoplasma* ([12]). In preparation to assess the impact of guanabenz on *in vivo* models of toxoplasmosis, we expanded *in vitro* testing to measure the effect of guanabenz on type II Prugniaud (Pru) parasites. For these studies, we selected a modified version of Pru containing a green fluorescent protein (GFP) reporter downstream of the LDH2 bradyzoite promoter, which provides a convenient means to monitor stage switching ([15]). Consistent with previous studies of strains RH and ME49 ([12]), guanabenz significantly inhibited replication of the Pru strain at concentrations as low as 1 μM, with a 50% effective concentration (EC_{50}) of 6 μM ([Fig. 1A]). We also determined if guanabenz could inhibit the growth of other apicomplexan parasites, such as *Plasmodium falciparum*, a causative agent of severe malaria. Using a conventional hypoxanthine incorporation assay ([23]), we found that guanabenz inhibited both strains HB3 (chloroquine sensitive) and Dd2 (chloroquine resistant), with EC_{50}s of 4.2 μM and 5.7 μM, respectively ([Fig. 1B]). Together, these studies show that guanabenz not only has activity against multiple *Toxoplasma* genetic backgrounds but also impedes replication of other apicomplexan parasites.

Guanabenz and salubrinal affect bradyzoite cysts generated *in vitro*. We previously found that guanabenz and salubrinal in-
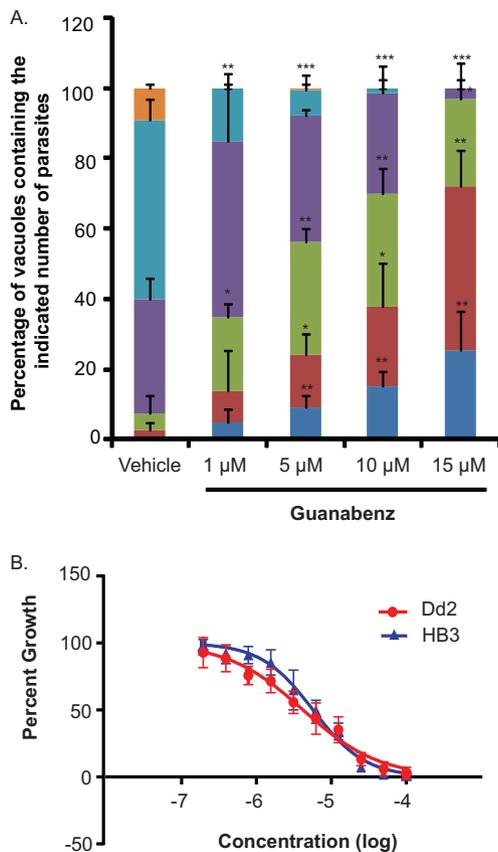
A.

B.

FIG 1 Guanabenz has potent activity against apicomplexan parasites *in vitro*. (A) Guanabenz inhibits the replication of the type II Prugniaud (Pru) strain *in vitro*. Tachyzoites were allowed to invade an HFF host cell monolayer for 4 h, at which point the uninvaded parasites were removed by replacing the medium with medium containing the indicated concentration of guanabenz or vehicle (saline). At 32 h postinfection, the numbers of parasites in 50 random vacuoles were determined and plotted as the percentages of the vacuoles containing the numbers of parasites shown (2, 4, 8, 16, etc.). Data represent the averages for 3 independent experiments. Statistical analysis was performed using the unpaired *t* test (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$). Error bars represent the averages and standard deviations for 3 independent experiments. (B) Guanabenz inhibits the replication of two independent strains of *Plasmodium falciparum in vitro* (Dd2 and HB3). Dose-response curves show the means for three biological replicates. The *y* axis shows the percent growth relative to that of vehicle controls, and the *x* axis shows the log of the concentration of guanabenz (GA).

hibited the reactivation of latent bradyzoites *in vitro*, but whether the drug treatment perturbed *Toxoplasma* cyst morphology was not determined (12). To investigate this question, tissue cysts were induced in HFF monolayers by using alkaline pH and CO_2 starvation for 6 days, at which point 15 μM guanabenz or 8 μM salubrinal was applied for 3 days (Fig. 2A). Inspection of drug-treated flasks revealed a subset of cysts that exhibited unusual morphologies under light microscopy. In sharp contrast to the densely packed and tightly organized bradyzoites present in normal cysts, some of the drug-treated cysts contained fewer, more loosely organized bradyzoites and an unusual amount of empty space (Fig. 2B). Additionally, *Dolichos* staining of the cyst wall was less robust in the abnormal cysts, indicating a degradation of cyst structures. To quantitate this phenomenon, 200 random cysts were examined and scored as normal or abnormal. Flasks treated

with vehicle or pyrimethamine never displayed any abnormal cysts, but 4% and 15% of cysts were abnormal in the guanabenz- and salubrinal-treated flasks, respectively. Attempts were made to amplify this effect by increasing the concentrations of guanabenz and salubrinal or the treatment duration, but this appeared to be detrimental to host cells. These results suggest that both guanabenz and salubrinal dually target tachyzoites and bradyzoites *in vitro*.

Guanabenz protects mice acutely infected with a lethal dose of *Toxoplasma*. Based on our *in vitro* findings, we sought to determine whether TgIF2α dephosphorylation inhibitors functioned against toxoplasmosis *in vivo*. We focused on guanabenz for these studies because it is already an FDA-approved drug and has excellent solubility and much better penetration into the central nervous system (CNS) than that of salubrinal (24, 25). Previously, we reported that guanabenz extends the life of BALB/c mice acutely infected with a lethal dose of hypervirulent RH strain tachyzoites: mice that normally succumb to infection by day 7 survived an additional 3 to 4 days when treated with the drug (12). Whether guanabenz is also effective against type II strains had not yet been determined. We therefore determined whether administration of guanabenz also promotes survival of mice infected with the ME49 or Pru strain. BALB/c mice were infected with a lethal dose of Pru or ME49 tachyzoites and administered guanabenz at 5 or 10 mg/kg of body weight or given the saline vehicle (Fig. 3). Drug treatment was initiated at 24 h postinfection and administered again every 48 h. Guanabenz prolonged the survival of mice infected with 10^6 Pru tachyzoites by 2 to 3 days, in a dose-dependent fashion (Fig. 3A). One hundred percent of mice infected with 10^4 ME49 tachyzoites and receiving the vehicle control succumbed to infection by day 9, but those treated with guanabenz displayed enhanced survival in a dose-dependent fashion (Fig. 3B). Sixty percent of infected mice receiving the higher dose of guanabenz survived the lethal inoculum of *Toxoplasma* ME49.

Reduced cyst burdens in mice treated with guanabenz during acute infection. Since type II strains readily form bradyzoites, a possible explanation for the protective effects of guanabenz could be enhanced tissue cyst formation *in vivo*. To address this possibility, we infected BALB/c mice with a sublethal dose of Pru strain tachyzoites and administered guanabenz (5 mg/kg/day) or vehicle for 19 days. Brain tissue cysts were visualized using *Dolichos* lectin staining and were enumerated as previously described (21). In the representative experiment shown in Fig. 4A, a 78% reduction of brain cysts was found in mice treated with guanabenz relative to those treated with vehicle. This experiment was repeated three independent times with similar results. In addition, we assessed the parasite burden in the brain by using qPCR to measure the *Toxoplasma* B1 gene DNA (22). Again, significantly fewer parasites were detected in mice receiving the guanabenz treatment (Fig. 4B).

Guanabenz reduces cyst burdens in chronically infected mice. Our experiments suggested that treatment with guanabenz can significantly lower the number of *Toxoplasma* tachyzoites *in vivo* without causing an increase in brain cyst burden. We next addressed whether guanabenz can affect brain cysts present in chronically infected mice. BALB/c mice were infected with a sublethal dose of Pru strain tachyzoites and allowed to develop chronic infection, with no drugs being administered. At day 25 postinfection, the chronically infected mice were treated with gua-

A.

$$Tz \xrightarrow[6\ d]{pH = 8.1} Bz \xrightarrow[8\ \mu M\ SAL]{DMSO\ 15\ \mu M\ GA} \text{Count 200}\ DB\ (+)\ cysts$$
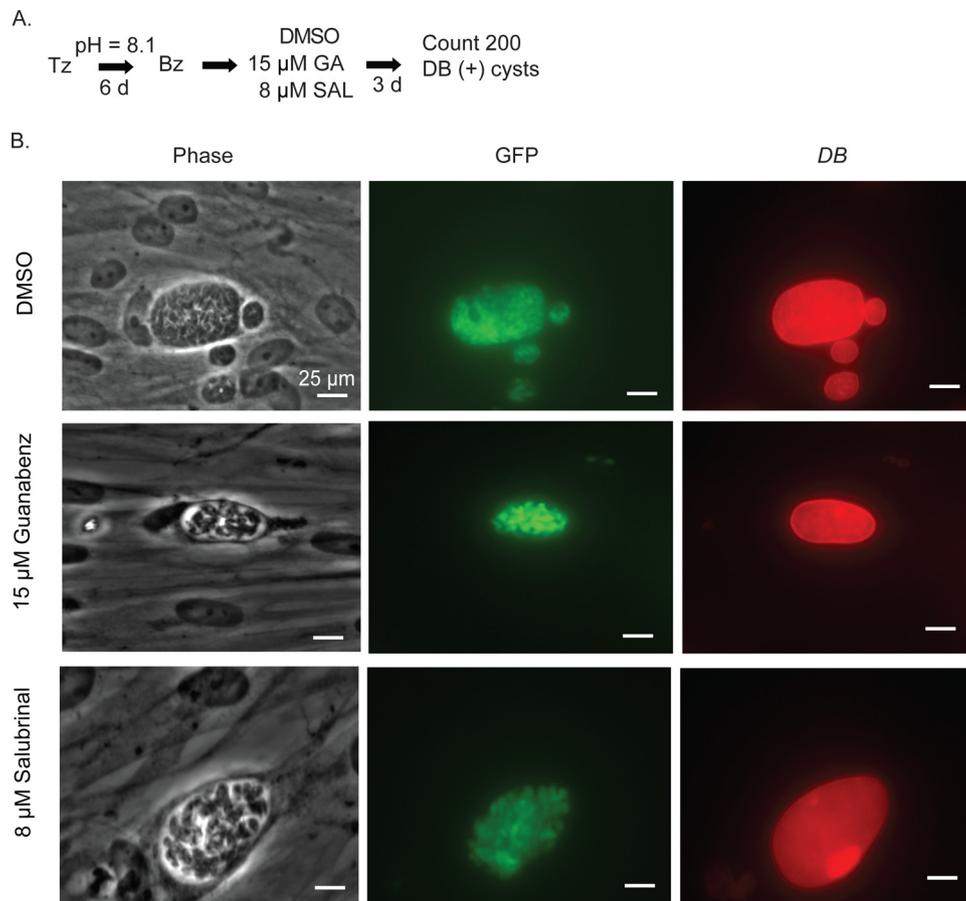
B.

| | Phase | GFP | DB |

FIG 2 Impacts of guanabenz and salubrinal on *in vitro* bradyzoite cysts. (A) Schematic representation of the experiment. Pru strain bradyzoite cysts were generated using alkaline medium and CO_2 starvation for 6 days. On day 6, guanabenz (GA), salubrinal (SAL), or vehicle (DMSO, the solvent for salubrinal) was added to the cultures, and tissue cysts were evaluated 3 days later. (B) Representative images of *in vitro*-generated cysts. The LDH2-GFP reporter (green) was used to visualize bradyzoites, and *Dolichos biflorus* lectin (DB; red) stain was used to observe cyst walls. Tz, tachyzoite; Bz, bradyzoite.

nabenz (5 mg/kg/day) or vehicle for 19 days, at which point the brains were harvested and tissue cysts counted. Strikingly, a significant reduction in cyst burden was found in the guanabenz-treated mice. Brains from the chronically infected mice receiving vehicle contained ~900 cysts, whereas those from guanabenz-treated mice contained only ~300 cysts, representing a 69% reduction in the number of cysts (Fig. 5). Similar reductions in the number of brain cysts were observed in drug-treated mice in three independent experiments. We concluded that treatment with guanabenz reduces the number of brain cysts in chronically infected mice.

DISCUSSION

New drugs are urgently needed to treat infections caused by apicomplexan parasites, such as *Toxoplasma* and *Plasmodium*. These parasites have complex life cycles that involve stages of latency and replication. In the case of *Toxoplasma*, latent bradyzoite tissue cysts thwart efforts to fully eradicate the infection from patients. An ideal drug should not only be effective against the proliferative stage of the parasite but also exert dual activity against the tissue cyst stage. We previously showed that translational control via the phosphorylation of TgIF2α is critical during both the tachyzoite and bradyzoite stages and is also important in

various aspects of *Plasmodium* physiology (9, 10, 26, 27). These findings suggest that pharmacological interference in translational control through parasite eIF2α may serve as a broad-spectrum strategy that targets multiple life cycle stages of different pathogens.

Two established inhibitors of TgIF2α dephosphorylation, guanabenz and salubrinal, have been shown to inhibit *Toxoplasma* replication and to impede the reconversion of bradyzoites into tachyzoites *in vitro* (12). In the present study, we significantly extended these findings to an *in vivo* context by using mouse models of acute and chronic toxoplasmosis. The present study focused on guanabenz for *in vivo* studies, as guanabenz is already an FDA-approved drug and has excellent solubility and much better penetration into the CNS than that of salubrinal (24, 25). Our results show that guanabenz not only protects mice from acute toxoplasmosis but also reduces the number of brain cysts in chronically infected animals. When *in vitro*-generated tissue cysts were treated with TgIF2α dephosphorylation inhibitors, the appearance of the bradyzoites was dramatically altered and the intensity of the cyst wall staining was diminished. No overt changes appeared in the host cells at these concentrations.

Concentrations of guanabenz are significantly higher in the brains of rats and mice than in the plasma (24), which is a likely
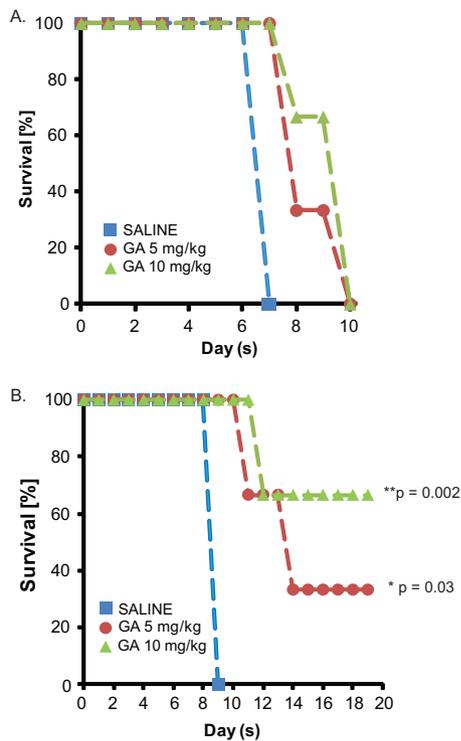
FIG 3 Efficacy of guanabenz (GA) for treatment of infection with a lethal dose of a *Toxoplasma* type II strain. (A) BALB/c mice were infected i.p. with a lethal dose (10^6) of type II Pru strain tachyzoites. Beginning 24 h later, guanabenz at 5 mg/kg or 10 mg/kg or vehicle (saline) was administered by i.p. injection, and this was repeated every 2 days. (B) BALB/c mice were infected with a lethal dose (10^4) of type II ME49 strain tachyzoites. Beginning 24 h later, the following treatments were administered and repeated every 2 days: guanabenz at 5 mg/kg or 10 mg/kg or vehicle (saline). For both panels, $n = 3$ for each group.

FIG 4 *Toxoplasma*-infected mice treated with guanabenz during acute infection have fewer brain cysts. Forty BALB/c mice were infected i.p. with a non-lethal dose (10^5) of Pru strain tachyzoites and randomized for subsequent treatments. Twenty mice were given guanabenz at 5 mg/kg/day, and the other 20 were treated with vehicle for 19 days, at which point brains were harvested so parasite burdens could be determined. Serological analyses confirmed infection in all mice, and 5 mice per group were randomly selected for analysis. (A) Direct counts of the number of cysts per brain in guanabenz-treated mice versus vehicle-treated mice. (B) qPCR analysis of infected brain samples, using the *Toxoplasma* B1 gene as an indicator of parasite burden. Statistical analysis was performed using the unpaired *t* test. Error bars represent the averages and standard deviations for cyst burden or parasite burden among the mice examined ($n = 5$ per group). Data shown are representative of 3 independent experiments that produced similar results.

contributing factor to the ability of the drug to reduce brain cysts. One study found that, on average, ~2 μM guanabenz can be detected in the brains of mice at 2 to 4 h postinjection (28), which corresponds to the concentration that we found to inhibit *Toxoplasma* proliferation in vitro (Fig. 1A). These findings are consistent with previous results showing that guanabenz and salubrinal can act directly on *Toxoplasma* to prevent TgIF2α dephosphorylation and that the antiparasitic activities of these drugs do not involve host eIF2α (12). However, it remains possible that guanabenz can affect host cell processes, such as those involving immunity, in ways that contribute to the observed activities against the parasite *in vivo*. Guanabenz was originally identified as an α2-adrenergic receptor agonist and has been used to treat hypertension for decades (29). More recently, guanabenz was shown to protect human cells from protein misfolding stress by binding to PPP1R15A/GADD34, thereby inhibiting eIF2α dephosphorylation (30). GADD34 is a regulatory subunit of the PP1 protein phosphatase complex that returns eIF2α to its unphosphorylated state. Interestingly, while guanabenz can clearly block TgIF2α dephosphorylation in a dose-dependent manner (12), there is no clear GADD34 gene orthologue evident in apicomplexan genome sequences. The precise mechanism by which guanabenz impairs TgIF2α dephosphorylation is an important issue to resolve in light of the findings presented here.

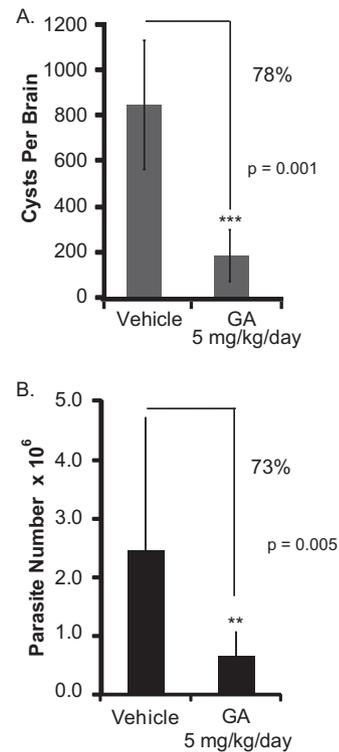Guanabenz (Wytensin) dosing for human patients taking the drug for hypertension scales from an initial dose of 4 mg orally twice a day to 8 mg orally twice a day, and patients typically use a maintenance dose of 4 to 16 mg orally twice a day. The maximum dose studied is 32 mg twice a day. While it is generally well tolerated, guanabenz is metabolized by the liver and should be used with caution in patients with liver impairment, and it should be noted that safety has not been established for patients of less than 18 years of age. The results reported here for mice show potent antiparasitic effects at a dose of 5 mg/kg once a day. While there are important caveats to consider for extrapolating human equivalent doses (HED) from animal studies (31), the HED for 5 mg/kg guanabenz in mice is 0.4 mg/kg, which is within the tolerated range of 0.07 to 0.53 mg/kg (4 to 32 mg given twice a day) for a 60-kg adult.

We also report here for the first time that guanabenz inhibits the replication of both chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum in vitro*, further highlighting the potential of guanabenz as a novel antiparasitic agent. Collectively, our study validates translational control mechanisms as viable new drug targets for treatment of apicomplexan infections.
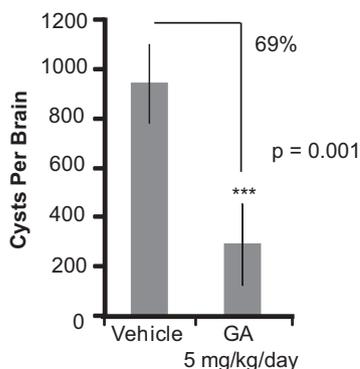
FIG 5 Guanabenz reduces the number of cysts in mice chronically infected with *Toxoplasma*. Ten BALB/c mice were infected i.p. with 10^5 Pru tachyzoites and allowed to progress to the chronic stage of disease for 25 days. On day 25, mice were randomized and divided into two groups, one receiving guanabenz at 5 mg/kg/day ($n = 5$) and the other receiving vehicle ($n = 5$). After 19 days of treatment, brains were harvested and cyst numbers determined. Serological analyses confirmed that all mice were infected. Data from a single representative experiment are shown, and two independent repeats produced similar results. Statistical analysis was performed using the unpaired *t* test, and error bars represent the averages and standard deviations for cyst burdens among the mice examined.

Because it is already FDA approved, guanabenz could rapidly be repurposed as an antiparasitic; however, newer derivatives of salubrinal and guanabenz are under intense investigation and should also be examined for activity against parasites. Very recently, a new guanabenz derivative, sephin 1, was described to be a potent inhibitor of eIF2α dephosphorylation (32). Sephin 1 lacked any measurable α2-adrenergic agonist activity in a cell-based assay, which may prove to be advantageous during prolonged treatment of parasite infection.

ACKNOWLEDGMENTS

REFERENCES

1. **Hill DE, Chirukandoth S, Dubey JP.** 2005. Biology and epidemiology of Toxoplasma gondii in man and animals. Anim Health Res Rev **6:**41–61. http://dx.doi.org/10.1079/AHR2005100.
2. **Sullivan WJ, Jr, Jeffers V.** 2012. Mechanisms of Toxoplasma gondii persistence and latency. FEMS Microbiol Rev **36:**717–733. http://dx.doi.org/10.1111/j.1574-6976.2011.00305.x.
3. **Jones J, Lopez A, Wilson M.** 2003. Congenital toxoplasmosis. Am Fam Physician **67:**2131–2138.
4. **Halonen SK, Weiss LM.** 2013. Toxoplasmosis. Handb Clin Neurol **114:**125–145. http://dx.doi.org/10.1016/B978-0-444-53490-3.00008-X.
5. **Zhang M, Joyce BR, Sullivan WJ, Jr, Nussenzweig V.** 2013. Translational control in Plasmodium and Toxoplasma parasites. Eukaryot Cell **12:**161–167. http://dx.doi.org/10.1128/EC.00296-12.
6. **Joyce BR, Konrad C, Wek RC, Sullivan WJ, Jr.** 2011. Translation control is critical during acute and chronic stages of toxoplasmosis infection. Expert Rev Anti Infect Ther **9:**1–3. http://dx.doi.org/10.1586/eri.10.146.
7. **Baird TD, Wek RC.** 2012. Eukaryotic initiation factor 2 phosphorylation and translational control in metabolism. Adv Nutr **3:**307–321. http://dx.doi.org/10.3945/an.112.002113.
8. **Hinnebusch AG.** 2014. The scanning mechanism of eukaryotic translation initiation. Annu Rev Biochem **83:**779–812. http://dx.doi.org/10.1146/annurev-biochem-060713-035802.
9. **Joyce BR, Queener SF, Wek RC, Sullivan WJ, Jr.** 2010. Phosphorylation of eukaryotic initiation factor-2{alpha} promotes the extracellular survival of obligate intracellular parasite Toxoplasma gondii. Proc Natl Acad Sci U S A **107:**17200–17205. http://dx.doi.org/10.1073/pnas.1007610107.
10. **Narasimhan J, Joyce BR, Naguleswaran A, Smith AT, Livingston MR, Dixon SE, Coppens I, Wek RC, Sullivan WJ, Jr.** 2008. Translation regulation by eukaryotic initiation factor-2 kinases in the development of latent cysts in Toxoplasma gondii. J Biol Chem **283:**16591–16601. http://dx.doi.org/10.1074/jbc.M800681200.
11. **Sullivan WJ, Jr, Narasimhan J, Bhatti MM, Wek RC.** 2004. Parasite-specific eukaryotic initiation factor-2 (eIF2) kinase required for stress-induced translation control. Biochem J **380:**523–531. http://dx.doi.org/10.1042/bj20040262.
12. **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. http://dx.doi.org/10.1128/AAC.01899-12.
13. **Holmes B, Brogden RN, Heel RC, Speight TM, Avery GS.** 1983. Guanabenz. A review of its pharmacodynamic properties and therapeutic efficacy in hypertension. Drugs **26:**212–229.
14. **Schafer SG, Kaan EC, Christen MO, Low-Kroger A, Mest HJ, Molderings GJ.** 1995. Why imidazoline receptor modulator in the treatment of hypertension? Ann N Y Acad Sci **763:**659–672. http://dx.doi.org/10.1111/j.1749-6632.1995.tb32460.x.
15. **Singh U, Brewer JL, Boothroyd JC.** 2002. Genetic analysis of tachyzoite to bradyzoite differentiation mutants in Toxoplasma gondii reveals a hierarchy of gene induction. Mol Microbiol **44:**721–733. http://dx.doi.org/10.1046/j.1365-2958.2002.02903.x.
16. **Roos DS, Donald RG, Morrissette NS, Moulton AL.** 1994. Molecular tools for genetic dissection of the protozoan parasite Toxoplasma gondii. Methods Cell Biol **45:**27–63.
17. **Coleman BI, Gubbels MJ.** 2012. A genetic screen to isolate Toxoplasma gondii host-cell egress mutants. J Vis Exp **8:**3807. http://dx.doi.org/10.3791/3807.
18. **Ferdig MT, Cooper RA, Mu J, Deng B, Joy DA, Su XZ, Wellems TE.** 2004. Dissecting the loci of low-level quinine resistance in malaria parasites. Mol Microbiol **52:**985–997. http://dx.doi.org/10.1111/j.1365-2958.2004.04035.x.
19. **Weiss LM, Kim K.** 2000. The development and biology of bradyzoites of Toxoplasma gondii. Front Biosci **5:**D391–D405. http://dx.doi.org/10.2741/Weiss.
20. **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. http://dx.doi.org/10.1073/pnas.1208069109.
21. **Lavine MD, Knoll LJ, Rooney PJ, Arrizabalaga G.** 2007. A Toxoplasma gondii mutant defective in responding to calcium fluxes shows reduced in vivo pathogenicity. Mol Biochem Parasitol **155:**113–122. http://dx.doi.org/10.1016/j.molbiopara.2007.06.004.
22. **Pina-Vazquez C, Saavedra R, Herion P.** 2008. A quantitative competitive PCR method to determine the parasite load in the brain of Toxoplasma gondii-infected mice. Parasitol Int **57:**347–353. http://dx.doi.org/10.1016/j.parint.2008.03.001.
23. **Chulay JD, Haynes JD, Diggs CL.** 1983. Plasmodium falciparum: assessment of in vitro growth by [3H]hypoxanthine incorporation. Exp Parasitol **55:**138–146. http://dx.doi.org/10.1016/0014-4894(83)90007-3.
24. **Meacham RH, Ruelius HW, Kick CJ, Peters JR, Kocmund SM, Sisenwine SF, Wendt RL.** 1980. Relationship of guanabenz concentrations in brain and plasma to antihypertensive effect in the spontaneously hypertensive rat. J Pharmacol Exp Ther **214:**594–598.
25. **Moreno JA, Radford H, Peretti D, Steinert JR, Verity N, Martin MG, Halliday M, Morgan J, Dinsdale D, Ortori CA, Barrett DA, Tsaytler P, Bertolotti A, Willis AE, Bushell M, Mallucci GR.** 2012. Sustained translational repression by eIF2α-P mediates prion neurodegeneration. Nature **485:**507–511. http://dx.doi.org/10.1038/nature11058.
26. **Zhang M, Fennell C, Ranford-Cartwright L, Sakthivel R, Gueirard P, Meister S, Caspi A, Doerig C, Nussenzweig RS, Tuteja R, Sullivan WJ, Jr, Roos DS, Fontoura BM, Menard R, Winzeler EA, Nussenzweig V.** 2010. The Plasmodium eukaryotic initiation factor-2alpha kinase IK2 controls the

latency of sporozoites in the mosquito salivary glands. J Exp Med **207:**1465–1474. http://dx.doi.org/10.1084/jem.20091975.

27. **Zhang M, Mishra S, Sakthivel R, Rojas M, Ranjan R, Sullivan WJ, Jr, Fontoura BM, Menard R, Dever TE, Nussenzweig V.** 2012. PK4, a eukaryotic initiation factor 2alpha(eIF2alpha) kinase, is essential for the development of the erythrocytic cycle of Plasmodium. Proc Natl Acad Sci U S A **109:**3956–3961. http://dx.doi.org/10.1073/pnas.1121567109.

28. **Way SW, Podojil JR, Clayton BL, Zaremba A, Collins TL, Kunjamma RB, Robinson AP, Brugarolas P, Miller RH, Miller SD, Popko B.** 2015. Pharmaceutical integrated stress response enhancement protects oligodendrocytes and provides a potential multiple sclerosis therapeutic. Nat Commun **6:**6532. http://dx.doi.org/10.1038/ncomms7532.

29. **Baum T, Shropshire AT.** 1976. Studies on the centrally mediated hypotensive activity of guanabenz. Eur J Pharmacol **37:**31–44. http://dx.doi.org/10.1016/0014-2999(76)90005-4.

30. **Tsaytler P, Harding HP, Ron D, Bertolotti A.** 2011. Selective inhibition of a regulatory subunit of protein phosphatase 1 restores proteostasis. Science **332:**91–94. http://dx.doi.org/10.1126/science.1201396.

31. **Reagan-Shaw S, Nihal M, Ahmad N.** 2008. Dose translation from animal to human studies revisited. FASEB J **22:**659–661.

32. **Das I, Krzyzosiak A, Schneider K, Wrabetz L, D'Antonio M, Barry N, Sigurdardottir A, Bertolotti A.** 2015. Preventing proteostasis diseases by selective inhibition of a phosphatase regulatory subunit. Science **348:**239–242. http://dx.doi.org/10.1126/science.aaa4484.