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 mis- configured, 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.
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% CO2.

To monitor Toxoplasma replication, a standard parasite counting assay was used (16). Briefly, 10⁶ Pru parasites were allowed to infect a confluent HFF monolayer. After 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 O3 blood red cells (Biocheomed, 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% O2, and 5% CO2. 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. [3H]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 × 10⁶ 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 CO2, 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 (10⁶ ME49 tachyzoites or 10⁵ 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 10⁶ 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% formaldehyde and stained with rhodamine-conjugated Dolichos biflorus lectin. For measurements of parasite burden, total genomic DNAs were isolated from brain homogenates by using DNase blood and tissue kits (Qiagen), and the Toxoplasma B1 gene was measured by quantitative PCR (qPCR) with the following primers: sense, 5’-GGAGGACCT GGCACACTGTTGCTCG; and antisense, 5’-TTGTTTTACCCGACGTTACAGG 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 Toxoplasma 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 (EC₅₀) 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 EC₅₀ 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-
Guanabenz Activity against Toxoplasma

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 ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 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).

hindered 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 CO2 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 TglF2α 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 10⁶ Pru tachyzoites by 2 to 3 days, in a dose-dependent fashion (Fig. 3A). One hundred percent of mice infected with 10⁴ 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-
nabenz (5 mg/kg/day) or vehicle for 19 days, at which point the
brains were harvested and tissue cysts counted. Strikingly, a sig-
nificant reduction in cyst burden was found in the guanabenz-
treated mice. Brains from the chronically infected mice receiving
vehicle contained /H11011900 cysts, whereas those from guanabenz-
treated mice contained only /H11011300 cysts, representing a 69% re-
duction 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 in-
fected mice.

DISCUSSION

New drugs are urgently needed to treat infections caused by api-
complexan 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 prolifer-
ative stage of the parasite but also exert dual activity against the
tissue cyst stage. We previously showed that translational con-
trol via the phosphorylation of TgIF2
/H9251is 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 dif-
ferent pathogens.

Two established inhibitors of TgIF2α dephosphorylation, gua-
nabenz 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 mod-
els 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 pen-
etration into the CNS than that of salubrinal (24, 25). Our results
show that guanabenz not only protects mice from acute toxoplas-
mosis but also reduces the number of brain cysts in chronically in-
fected mice.

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 CO2 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.
adrenergic receptor agonist and has been used to treat hypertension. Interestingly, while guanabenz can clearly block TgIF2 phosphorylation in a dose-dependent manner (12), there is no phosphatase dose of a PPP1R15A/GADD34, thereby inhibiting eIF2 dephosphorylation.

Guanabenz activity against Toxoplasma

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.
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 and should also be examined for activity against parasites. Very 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.


