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Rebound of Cyst Number Following Discontinuation of Guanabenz Treatment for Latent Toxoplasmosis

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Abstract

Toxoplasma gondii is a protozoan parasite that causes opportunistic infection in immunocompromised individuals. The parasite forms latent tissue cysts that are refractory to current treatments and give rise to life-threatening reactivated infection following immune suppression. Previously, we showed that guanabenz sharply reduces brain cyst count in BALB/c mice harboring latent toxoplasmosis; however, whether cyst count would change once drug treatment stopped was not addressed. In the present study, we observed a rebound in brain cysts following the discontinuation of guanabenz or a guanabenz-pyrimethamine combination therapy. The re-expansion of brain cysts was not accompanied by symptoms of acute toxoplasmosis. We also tested whether the rebound in cyst counts could be ameliorated by administering pyrimethamine during or after guanabenz treatment.

Keywords

guanabenz; parasites; Toxoplasma; host-pathogen interactions; anti-infective drugs

Results and Discussion

Toxoplasma gondii is an obligate intracellular parasite that causes opportunistic infections in immune suppressed individuals (1–3). Upon infection with *Toxoplasma*, the rapidly replicating tachyzoites (doubling time ~6–10 hours) convert into quiescent bradyzoites that are housed in tissue cysts. Opportunistic toxoplasmosis arises from reactivation of a

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Author Statement

Individual contributions to the paper using the relevant CRediT roles:

Jennifer Martynowicz: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing.

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previous infection, in which the bradyzoites reconvert into proliferative tachyzoites that can cause tissue damage to critical organ systems (4, 5). Current antifolate treatments are only effective against actively dividing tachyzoites due to its effects on nucleic acid synthesis; at-risk individuals may be placed on prophylactics for the duration of their immunosuppression, which can last the rest of their lives (2, 5–8). These therapies are accompanied by a high rate of adverse side effects that often force discontinuation of treatment (5).

Guanabenz has potent activity against *Toxoplasma* tachyzoites and tissue cysts, suggesting that it could be repurposed as an anti-parasitic agent (9–12). BALB/c mice chronically infected with *Toxoplasma* showed a ~75% reduction in brain cysts following a three-week course of guanabenz, but it remains unknown if the remaining cysts are capable of repopulating the brain after drug treatment ends (10). In this study, we found that brain cyst counts rebound after cessation of guanabenz treatment and examined whether this re-expansion of cysts could be reduced by pyrimethamine.

Prolonged guanabenz treatment (up to 6 weeks) does not further reduce brain cyst count beyond the ~75% decrease seen with a 3 week course (10). Since these remaining cysts could be attenuated or nonviable, we sought to examine whether brain cyst counts would rise after guanabenz treatment was stopped. Female BALB/c mice harboring latent toxoplasmosis were given guanabenz for three-weeks and then given vehicle alone for 3 weeks (Fig. 1A). No adverse events indicative of widespread reactivated infection accompanied the cessation of guanabenz in these mice; on the contrary, the relative weight of the treatment groups improved with the stoppage of guanabenz (Fig. 1B). It is important to note, however, that guanabenz-treated mice tend to have relatively lower weights than vehicle controls whether they are infected or not (data not shown), so the relatively lower weights in the guanabenz treatment groups is not unusual. As seen previously (9, 10), 3 weeks of guanabenz significantly reduces brain cyst count; however, discontinuation of guanabenz results in a rebound of brain cysts within 3 weeks (Fig. 1C).

Interestingly, mice that experienced a rebound in brain cyst counts showed no symptoms of reactivated infection, suggesting that active infection with tachyzoites could be minimal. The new cysts could be formed by low levels of reactivated tachyzoites, ruptured cysts, dividing bradyzoites that escape its original cyst, or a combination of these events. We therefore tested if pyrimethamine, which targets actively replicating parasites, could prevent the re-expansion of brain cysts after guanabenz is withdrawn. Chronically infected female BALB/c mice were treated with either guanabenz or vehicle for 3 weeks and then given either vehicle, guanabenz, or pyrimethamine for an additional 3 weeks (Fig. 2A). As before, none of the mice displayed symptoms of reactivation; the only group that did not regain weight was the one receiving guanabenz for the entire 6 weeks (Fig. 2B). As we observed in Fig. 1, discontinuation of guanabenz without additional therapy resulted in restoration of cyst count to levels resembling untreated controls (Fig. 2C). As expected, mice that did not receive guanabenz showed high cyst count regardless of pyrimethamine administration. Mice receiving pyrimethamine after guanabenz treatment resembled the group receiving guanabenz for 6 weeks, and maintained a significantly lower cyst count than untreated controls. These results suggest that, following the discontinuation of guanabenz, actively

dividing parasites repopulate the brain and pyrimethamine may impede this process. Further studies are warranted to assess if other drugs targeting parasite replication can significantly curtail this re-expansion of cysts.

We next sought to determine if treatment of chronic infection with a combination of guanabenz-pyrimethamine for 3 weeks would prevent cyst rebound after its discontinuation. In addition, we examined the effect of continued pyrimethamine after completion of the guanabenz-pyrimethamine course. Mice with latent *Toxoplasma* infections were treated with either vehicle or guanabenz-pyrimethamine for 3 weeks (Fig. 2D). The vehicle group was maintained on vehicle for an additional 3 weeks while the drug combination group was subdivided into 3 treatment groups: continued guanabenz-pyrimethamine for 3 weeks, pyrimethamine for 1 week followed by vehicle for 2 weeks, or vehicle for 3 weeks (Fig. 2D).

We observed the same weight pattern as observed previously. The mice receiving guanabenz trend at a lower weight, but immediately regain it upon discontinuation of guanabenz, regardless of the inclusion of pyrimethamine (Fig. 2E). Guanabenz-pyrimethamine treatment reproduced the significant decrease in brain cyst count as we recently reported (Fig. 2F) (12). However, discontinuation of this combination drug treatment results in a rebound of cyst numbers back to levels seen in untreated mice, similar to what was observed with discontinuation of guanabenz monotherapy (Fig. 1C). Prolonging pyrimethamine treatment for 1 week after discontinuing the guanabenz-pyrimethamine treatment was not sufficient to show a statistically significant difference in brain cyst counts (Fig. 2F).

Guanabenz has a remarkable ability to diminish brain cyst number in BALB/c mice chronically infected with *Toxoplasma*, but appears unable to eliminate the cysts completely (9–12). We show here that these residual cysts, perhaps located in areas of the brain or body that are inaccessible to guanabenz, could be a potential source of parasites that repopulate the brain after discontinuation of guanabenz. Curiously, the re-expansion of cyst numbers in the brain occurs without physical symptoms of reactivated infection (i.e weight loss, tremors, seizures, death). Pyrimethamine trended toward impeding the re-expansion, but results were not statistically significant. Future studies exploring combinations of cyst-reducing drugs and more robust drugs that stop parasite replication are warranted in the effort to eradicate latent parasites. We chose to examine a guanabenz dose of 5mg/kg/day, as 10mg/kg/day did not show greater efficacy in a previous study (9); however, higher doses of guanabenz may prove more useful in the context of synergistic drugs.

It should be noted that there is inherent variability in the mouse models of chronic toxoplasmosis, often producing large error bars for cyst counts. Additional repeats of these studies are warranted to build confidence in the results, although it is important to stress that guanabenz has consistently reduced cyst numbers in BALB/c mice across multiple independent studies (9–10, 12). Given its efficacy against tissue cysts, it is of interest to characterize more extensively the impact of guanabenz treatment on tissue cyst size and bradyzoite viability in vivo. Furthermore, since toxoplasmosis often occurs during immune suppression, it would be of value to examine the effect of guanabenz on reactivated infection using immunocompromised mouse models of latent toxoplasmosis.

Methods

Parasite strains and culture

Toxoplasma gondii parasites (Type II Prugniaud (Pru) strain) were collected as tissue cysts from brains of chronically infected BALB/c mice and used to infect human foreskin fibroblasts (HFF). 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 no more than 15 passages. Parasites were tested for mycoplasma using PCR as previously described (13) prior to use in mice.

Mouse strains and infection

The mice used in this study were housed in American Association for 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 Indiana University School of Medicine approved the use of all animals and procedures (IACUC protocol numbers 10852 and 11376).

BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME) at 5 weeks old. The mice were allowed to acclimate for one week before being intraperitoneally (i.p.) infected with 10^4 Pru tachyzoites suspended in 100 μl of autoclaved, filter-sterilized PBS. Mice were routinely observed multiple times a day throughout the course of acute infection. After 21 days post-infection, mice were randomized into treatment groups. Blood samples were extracted by cardiac puncture following euthanasia to confirm parasite infection through serological analysis of 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 1.5mL Eppendorf tubes and stored at 4°C until needed. Guanabenz (Sigma) was delivered i.p. 5mg/kg/day in sterile saline at a volume of 50 μl. Pyrimethamine (MP Biomedicals) was dosed i.p. at 20mg/kg/day in sterile saline at a volume of 50 μl as previously reported (9, 12).

Brain Cyst Quantification

Brains were extracted upon euthanasia and cyst count was quantified as previously described (9). 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 minutes. 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 sealed before imaging. Stained samples were blinded before cysts were counted under 20x magnification. The counted value was then extrapolated to estimate the cyst count for the entire brain.

Statistics

Statistical analysis was performed using GraphPad Prism version 7.03. For datasets comparing two groups, statistical significance was determined using Student's t test. If more than two groups were compared, the datasets were analyzed using One Way AVOVA to determine statistical significance before secondary analysis with Student's t test. P values of ≤ 0.05 were considered statistically significant.

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Highlights

- We provide further evidence that guanabenz significantly reduces the number of latent *Toxoplasma gondii* tissue cysts in the brains of BALB/c mice.
- We examined brain cyst counts after cessation of guanabenz treatment and found that they rebound without causing symptoms of acute toxoplasmosis.
- We also tested whether the re-expansion of tissue cysts could be impeded by administering pyrimethamine during or after guanabenz treatment.

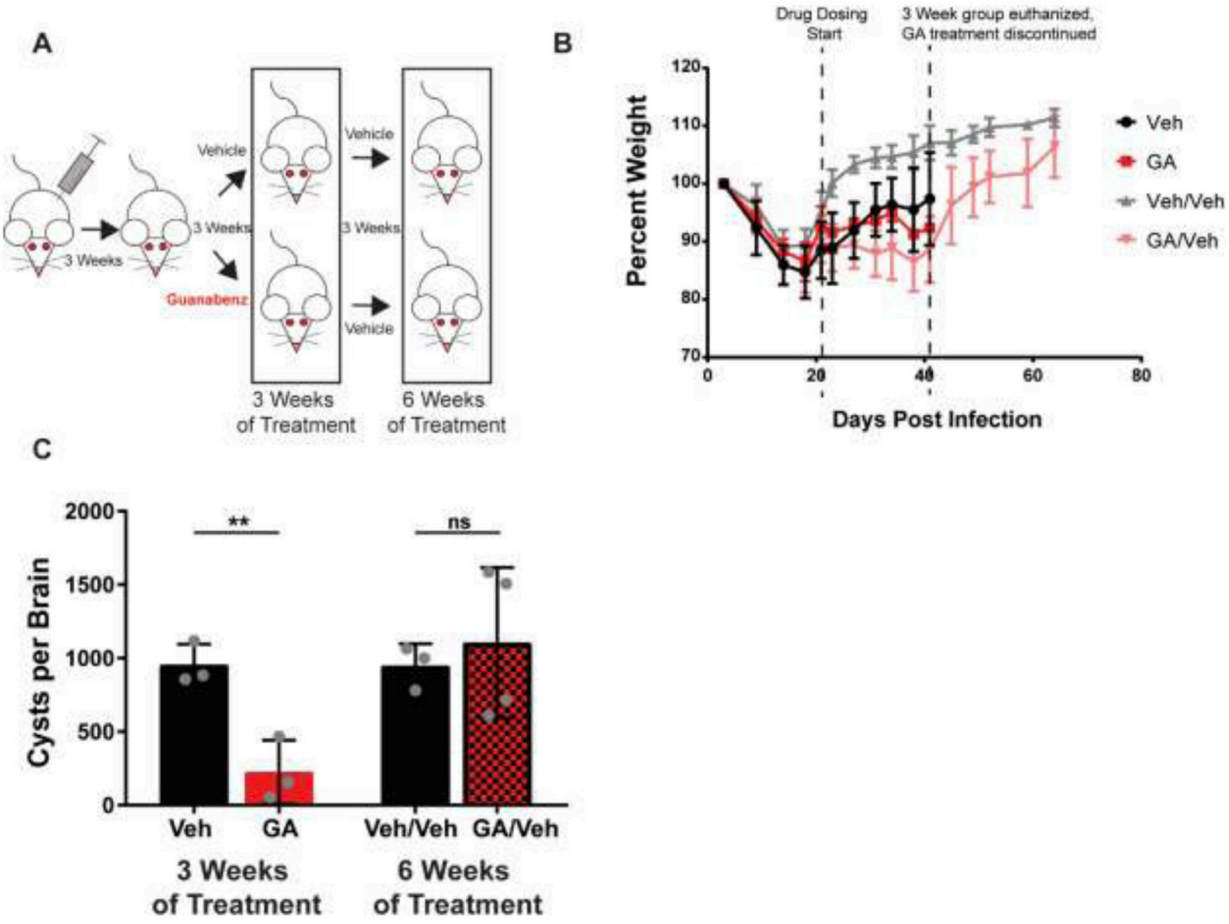

Figure 1. Discontinuation of guanabenz causes rebound in cyst count in BALB/c mice.

(A) Six-week old, female BALB/c mice were infected intraperitoneally with *Toxoplasma* (grey syringe) and allowed to progress to chronic infection for 3 weeks. The mice were then randomized into two different treatment groups, receiving either guanabenz (GA) or vehicle (Veh). After 3 weeks (42 days post-infection), GA was stopped and replaced with vehicle for 3 weeks (63 days post-infection); the vehicle group remained on vehicle for 3 weeks longer (N=3 or 4). (B) Mice were weighed every 2–3 days and the percent weight was calculated with the day of infection as 100%. (C) Following 3 weeks of treatment, brain cyst count was blindly quantified. One way ANOVA ($p=0.0324$) was followed by an unpaired Student's t test. ns=not significant, $^{**}p<0.01$.

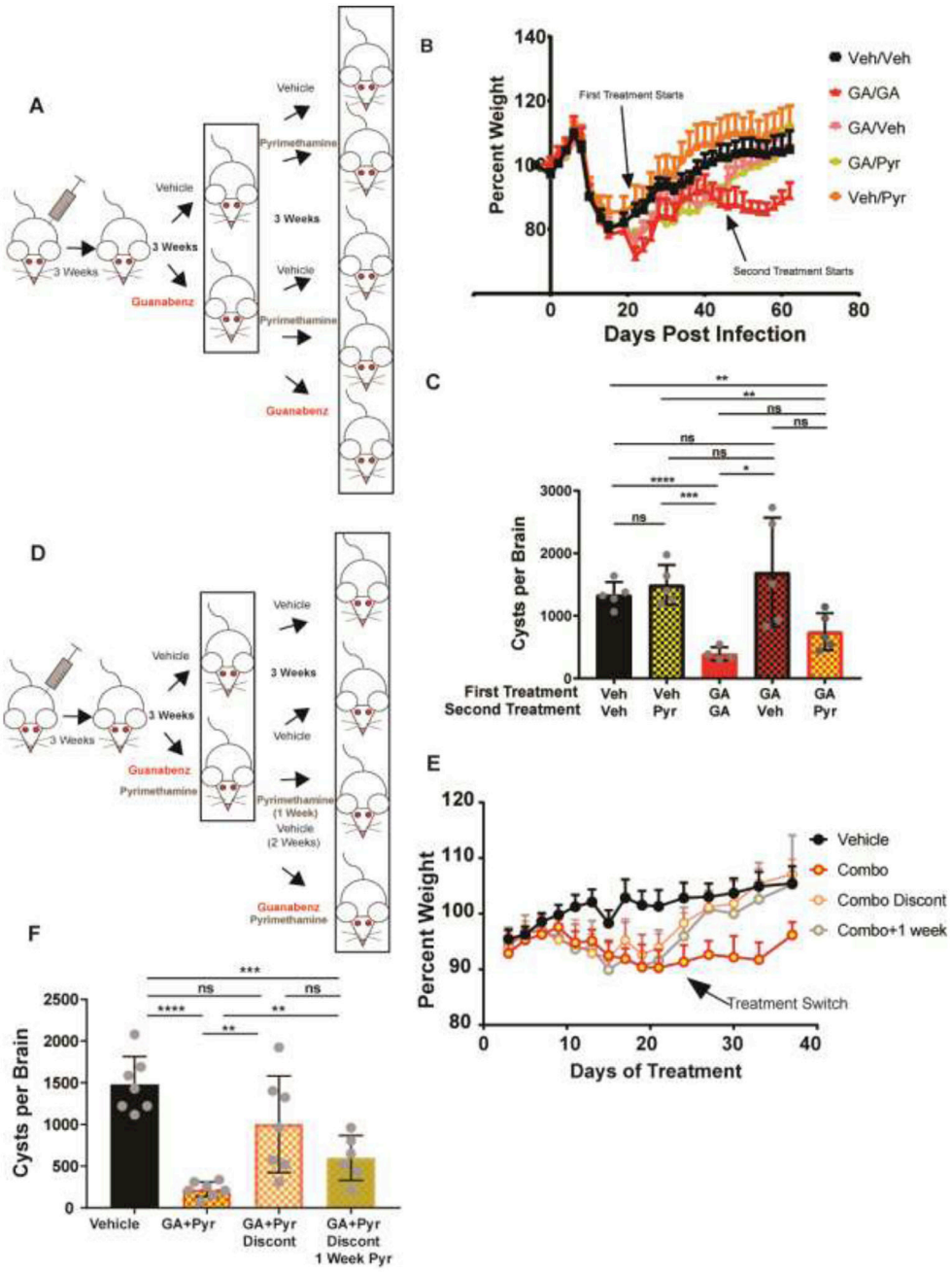

Figure 2. Pyrimethamine impedes re-expansion of cyst number following withdrawal of guanabenz.

(A) Six-week old, female BALB/c mice were infected intraperitoneally with *Toxoplasma* (grey syringe) and allowed to progress to chronic infection for 3 weeks. The mice were then randomized into two different treatment groups for 3 weeks and then subdivided into 5 indicated treatment groups (N=5). (B) Mice were weighed every 2–3 days and the percent weight was calculated with the day of infection as 100%. (C) Following the 6 total weeks of treatment, brain cyst count was blindly quantified. One way ANOVA p=0.0020 followed by an unpaired Student's t test. ns=not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. (D) Six-week old, female BALB/c mice were infected intraperitoneally with

Toxoplasma (grey syringe) and allowed to progress to chronic infection for 3 weeks (21 days post-infection). The mice were then randomized into two different treatment groups for 3 weeks and subdivided into 4 treatment groups for 3 more weeks (42 days post-infection) (N=7). (E) Mice were weighed every 2–3 days and the percent weight was calculated with the day of infection as 100%. (F) Following 3 weeks of treatment, brain cyst count was blindly quantified. The second set of mice were treated for three more weeks before cyst count quantification. One way ANOVA $p<0.0001$ followed by an unpaired Student's t test. ns=not significant, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.