

# Inhibitors of eIF2 $\alpha$ Dephosphorylation Slow Replication and Stabilize Latency in *Toxoplasma gondii*

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*Toxoplasma gondii* is an obligate intracellular parasite that permanently infects warm-blooded vertebrates through its ability to convert into a latent tissue cyst form. The latent form (bradyzoite) can reinitiate a life-threatening acute infection if host immunity wanes, most commonly in AIDS or organ transplant patients. We have previously shown that bradyzoite development is accompanied by phosphorylation of the parasite eukaryotic initiation factor 2 alpha subunit (eIF2 $\alpha$ ), which dampens global protein synthesis and reprograms gene expression. In this study, we analyzed the activities of two specific inhibitors of eIF2 $\alpha$  dephosphorylation, salubrinal (SAL) and guanabenz (GA). We establish that these drugs are able to inhibit the dephosphorylation of *Toxoplasma* eIF2 $\alpha$ . Our results show that SAL and GA reduce tachyzoite replication *in vitro* and *in vivo*. Furthermore, both drugs induce bradyzoite formation and inhibit the reactivation of latent bradyzoites *in vitro*. To address whether the antiparasitic activities of SAL and GA involve host eIF2 $\alpha$  phosphorylation, we infected mutant mouse embryonic fibroblast (MEF) cells incapable of phosphorylating eIF2 $\alpha$ , which had no impact on the efficacies of SAL and GA against *Toxoplasma* infection. Our findings suggest that SAL and GA may serve as potential new drugs for the treatment of acute and chronic toxoplasmosis.

The protozoan parasite *Toxoplasma gondii* (phylum Apicomplexa) is an important life-threatening opportunistic pathogen in AIDS patients and other immunosuppressed individuals (1). During the course of infection, the host immune response induces this obligate intracellular parasite to convert from a proliferating stage (tachyzoite) into a latent tissue cyst stage (bradyzoite). Bradyzoite cysts can remain in host tissues for life, seemingly without overt health consequences. However, bradyzoites can revert to actively replicating tachyzoites when host immunity is attenuated, causing lifelong chronic infection. The recurrent reactivation of acute infection can lead to serious damage of the central nervous system (CNS) and heart, where bradyzoite cysts have a tendency to reside (1–3). While antifolates and other antibiotics are used to treat acute infection, the frontline drugs cause severe adverse effects and do not appear to exert activity against encysted parasites (1, 3, 4). Thus, there is an urgent need for new therapeutics to treat acute and latent toxoplasmosis.

The molecular mechanisms coordinating stage conversion are poorly understood. Tachyzoite-to-bradyzoite conversion can be induced *in vitro* by a range of different stresses, including pH extremes, heat shock, and exposure to sodium nitroprusside or mitochondrial inhibitors (3). Removal of the stress condition prompts the encysted bradyzoites to revert into proliferative tachyzoites. We previously showed that bradyzoite conversion triggered by exposure to different stress conditions is accompanied by phosphorylation of the alpha subunit of *Toxoplasma* eukaryotic initiation factor 2 (TgIF2 $\alpha$ ) (5, 6). The eukaryotic initiation factor 2 alpha subunit (eIF2 $\alpha$ ) is phosphorylated by a specialized group of protein kinases that possess a signature catalytic domain flanked by distinct regulatory domains, the latter of which allow their activation by disparate environmental stresses or signals (7). Phosphorylation of eIF2 $\alpha$  results in a dramatic reduction in global protein synthesis, allowing cells time to organize a new pattern of gene expression designed to remedy the stress or undergo a developmental change (7). We identified four eIF2 $\alpha$  kinases in *Toxoplasma* and confirmed that TgIF2 $\alpha$  phosphoryla-

tion results in translational control during stress (5, 8–10). We also found that TgIF2 $\alpha$  phosphorylation is maintained in mature bradyzoites, consistent with the dormant nature of this life cycle stage (6). These findings support the idea that TgIF2 $\alpha$  phosphorylation could represent a potential new target for the treatment of *Toxoplasma* infection (8, 11).

During a screen for small molecules that protect rat PC12 cells from endoplasmic reticulum (ER) stress, salubrinal (SAL) was found to prevent host cell apoptosis by specifically preventing dephosphorylation of mammalian eIF2 $\alpha$  (12). In a previous study, we found that treatment of *Toxoplasma* tachyzoites with SAL leads to TgIF2 $\alpha$  phosphorylation and induction of the BAG1 and LDH2 bradyzoite-specific marker genes (6). More recently, guanabenz (GA), an  $\alpha$ 2-adrenergic receptor agonist used in the treatment of hypertension (13), has also been found to promote survival of human HeLa cells upon ER stress through inhibition of eIF2 $\alpha$  dephosphorylation (14). In light of our previous work linking TgIF2 $\alpha$  phosphorylation and microbial latency, we sought to determine the impact of eIF2 $\alpha$  dephosphorylation inhibitors on *Toxoplasma* stage conversion *in vitro*. Results from this study suggest that the drugs SAL and GA inhibit *Toxoplasma* infection by a mechanism involving induced translational control that promotes and stabilizes the latent bradyzoite form. Consistent with the slowed growth observed *in vitro*, GA was also able to extend the life of mice infected with a hypervirulent strain of *Toxoplasma*.

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## MATERIALS AND METHODS

**Chemicals.** Salubrinal (SAL) was purchased from Tocris Bioscience (no. 2347). Guanabenz acetate (GA) (no. G110) and clonidine (CLN) (no. C7897) were purchased from Sigma-Aldrich.

**Parasite cultivation.** *Toxoplasma* tachyzoites were cultivated in human foreskin fibroblasts (HFFs) in Dulbecco's modification of Eagle's medium (DMEM) (Mediatech) containing 25 mM glucose and 4 mM glutamine and supplemented with 1% heat-inactivated fetal bovine serum (FBS) (Gibco/Invitrogen). Cultures were maintained in a humidified incubator at 37°C with 5% CO<sub>2</sub>. For the cultivation and infection of mouse embryonic fibroblast (MEF) cells, DMEM was supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin.

**Parasite proliferation assays.** Ten thousand tachyzoites were allowed to infect confluent HFF cells grown on coverslips in DMEM plus 1% FBS. At 4 h postinfection, infection medium was replaced, supplemented with the indicated concentration of SAL, GA, CLN, or vehicle (dimethyl sulfoxide [DMSO]). To determine the number of tachyzoites per vacuole, infected HFFs were fixed with 0.5 ml ice-cold methanol for 15 min at -20°C and nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) to facilitate counting of parasites by fluorescence microscopy in at least 50 random vacuoles.

For experiments using MEF cells, ~50,000 MEF cells were seeded on glass coverslips in 12-well plates for approximately 16 to 20 h prior to infection with 25,000 ME49 tachyzoites in DMEM plus 10% FBS supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin. At 4 h postinfection, infection medium was replaced with medium supplemented with the indicated concentrations up to 8 µM SAL, 15 µM GA, or vehicle (DMSO), as indicated. In mammalian cells, SAL and GA were reported to be nontoxic at concentrations of up to 100 µM and 50 µM, respectively (12, 14). In contrast to these reports, we observed toxic effects of GA on HFFs at concentrations higher than 25 µM, which could be due to the extended incubation time of up to 7 days.

At the indicated time points, cells were fixed in 3% paraformaldehyde for 30 min at room temperature. Following incubation in 1× phosphate-buffered saline (PBS) supplemented with 0.1 M glycine, samples were then permeabilized for 20 min with 1× PBS supplemented with 0.2% Triton X-100 and 3% bovine serum albumin (BSA) (fraction V; Omnipur) and subsequently blocked for 1 h with 3% BSA in 1× PBS. To visualize tachyzoites for counting, samples were incubated for 1 h with a monoclonal mouse anti-Sag1 antibody (1:5,000, C86319M; Meridian Life Science) in blocking solution, followed by incubation for 1 h with a goat anti-mouse antibody (1:2,000, A11029; Molecular Probes) conjugated with Alexa 488 (1:2,000, A11029; GE Healthcare). All incubation steps were carried out at room temperature.

To determine the number of parasites using the PCR-based B1 assay, MEF cells were seeded in 12-well plates and infected as described above. At 4 h and 24 h postinfection, infection medium was replaced, with supplementation with the indicated concentrations of SAL, GA, or vehicle (DMSO). At 48 h postinfection, genomic DNA was isolated from the infected cells, and parasite counts were determined using quantitative PCR with primers for the *Toxoplasma* B1 gene as described previously (15).

**Monitoring tachyzoite-bradyzoite interconversion.** Five hundred ME49 strain tachyzoites were inoculated onto confluent HFF monolayers grown on glass coverslips. Four hours postinfection, culture medium was replaced with alkaline medium (pH 8.1), which was replaced daily. After 7 days in alkaline stress medium, infected monolayers were washed and incubated in normal medium (pH 7.2) plus SAL, GA, or vehicle (DMSO) to induce reconversion into tachyzoites. A parallel sample was maintained in alkaline medium to prevent reactivation. All media were replaced daily. When infection resumed in the vehicle control (as determined by host cell lysis observed by light microscopy, usually 3 to 4 days after switching from alkaline to normal medium), infected monolayers were fixed in ice-cold methanol, and bradyzoite cysts were visualized by indirect fluorescent-antibody assay (IFA) following staining with fluorescein isothiocyanate

(FITC)-conjugated *Dolichos biflorus* lectin (1:200, FL-1031; Vector Laboratories) as described previously (6).

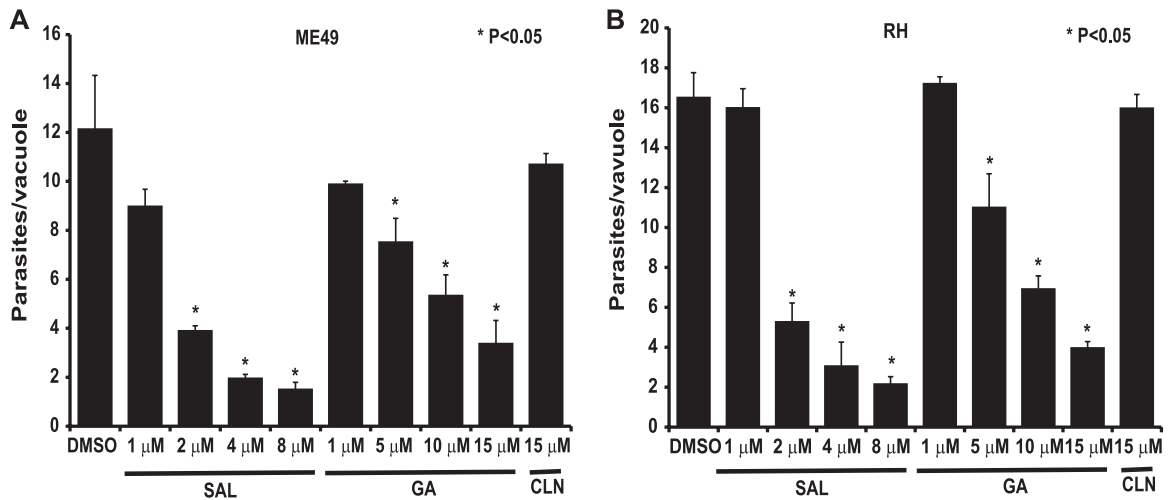
To determine the number of tachyzoites released into the medium upon reactivation of infection, confluent HFF monolayers grown in T-25 25-cm<sup>2</sup> tissue culture flasks were infected with 10<sup>6</sup> ME49 tachyzoites and converted into bradyzoites using the alkaline pH method described above. At day 8, alkaline medium was washed out and replaced with normal medium supplemented with the appropriate concentrations of SAL, GA, or vehicle (DMSO), and replaced daily to induce reactivation of infection. After 3 to 4 days, the reactivated tachyzoites present in the culture supernatant were counted using a Neubauer hemacytometer.

To measure induced expression of bradyzoite marker genes upon treatment with GA, confluent HFF monolayers were grown in T-25 25-cm<sup>2</sup> tissue culture flasks and infected with ~10<sup>6</sup> ME49 tachyzoites. Four hours postinfection, the infection medium was replaced with medium supplemented with 15 µM GA or vehicle (DMSO) and replaced daily. After 5 days, intracellular parasites were released by syringe passage and washed twice with ice-cold PBS. Total RNA was isolated using an RNeasy Plus kit (Qiagen). cDNA was synthesized using an oligo(dT) primer (Qiagen) and the Omniscript kit (Qiagen). Quantitative PCR was carried out as described previously (6) using the following gene-specific oligonucleotides: β-tubulin (for normalization), sense (ATGTTCCGTGGTCCG ATGT) and antisense (TGGGAATCCACTGAACGAAGT); LDH2, sense (TGAGGAACGTGTGGTCCACAGA) and antisense (TTGGTAACCCCGTGAAAAA); BAG1, sense (TGAGCGAGTGTCCGGTTATTT) and antisense (TAGAACGCCGTTGTCCATTG).

**Recovery of bradyzoites from SAL and GA treatment.** As a measure of parasite infection of host cells following removal of alkaline stress, 500 ME49 strain tachyzoites were inoculated onto confluent HFF monolayers grown in 12-well plates and treated as described above. After 7 days, infected monolayers were washed and incubated in normal medium supplemented with the indicated concentration of SAL or GA and replaced daily. For controls, one set of samples was maintained in alkaline medium. After another 3 days (10 days postinfection), all infected monolayers were incubated in normal medium. One week later (17 days postinfection), host cells were fixed with methanol and stained with crystal violet. The degree of host cell lysis was determined with an Alpha Innotech imager (MultiImage II, AlphaView software, version 2.0.0.9).

**Inhibition of TgIF2α dephosphorylation in the presence of SAL and GA.** ME49 tachyzoites were physically released from host cells via syringe passage. Filter-purified parasites were incubated in DMEM plus 1% FBS supplemented with 10 µM thapsigargin (T9033; Sigma) or vehicle (DMSO) for 1 h. Following a wash step, parasites were incubated for another hour in DMEM plus 1% FBS supplemented with the indicated concentration of SAL, GA, or vehicle (DMSO). All incubation steps were carried out at 37°C and 5% CO<sub>2</sub>. Western blot analysis of TgIF2α phosphorylation was carried out as previously described using polyclonal rabbit antibodies recognizing either total TgIF2α or TgIF2α phosphorylated at serine 71 (5, 6).

**In vivo treatment of acute infection.** BALB/c mice were purchased from Harlan Laboratories (Indianapolis, IN) and infected via intraperitoneal (i.p.) injection with 100 tachyzoites of the RH strain, isolated via syringe passage from an HFF monolayer and resuspended in sterile 1× PBS. A total of 30 mice, which were separated into 5 groups consisting of 6 mice each (3 per cage), were used in the study. The tachyzoite suspension was kept at 37°C until injection (groups 1 to 4). Control mice were injected with a corresponding volume of 1× PBS (group 5). At 4 h postinfection, mice in group 1 were injected with vehicle, group 2 with 5 mg/kg of body weight GA, and groups 3 to 5 with 10 mg/kg GA. Group 1 received twice-daily injections of vehicle until the end of the study. For groups 2 and 3, this regimen was continued until mice were moribund (group 2, day 7/group 3, day 10). Groups 4 and 5 received injections of 10 mg/kg GA on day 2, and beginning on day 3, they were administered a total of 20 mg/kg GA in two injections of 10 mg/kg each (separated by 10 to 12 h), which was administered until the end of the study. GA was dissolved at a



**FIG 1** Salubrinal (SAL) and guanabenz (GA) reduce replication of *Toxoplasma* *in vitro*. HFF host cells infected with ME49 (A) or RH (B) strain parasites were cultured with the indicated concentration of SAL, GA, clonidine (CLN), or vehicle (DMSO). At 32 h postinfection, replication was assessed by counting tachyzoites in at least 50 random vacuoles. Histograms show averages for a representative experiment performed in triplicate. Error bars indicate standard deviations, and asterisks indicate a statistically significant difference relative to results for the vehicle control ( $P < 0.05$ , two-tailed unpaired *t* test).

concentration of 2.5 mg/ml in physiological salt solution and sterile filtered prior to i.p. injection. Wet food was provided within the cage throughout the study. Mice were weighed before injection, and the injection volume was adjusted accordingly. All mice were checked twice daily for signs of infection and euthanized when moribund. Peritoneal fluid was analyzed microscopically for the presence of tachyzoites.

## RESULTS

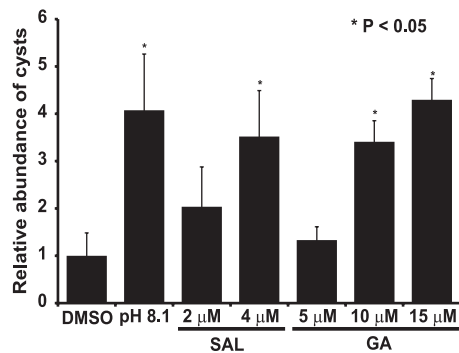
**Salubrinal and guanabenz inhibit tachyzoite replication *in vitro*.** In a previous study, we showed that SAL can inhibit dephosphorylation of TgIF2 $\alpha$  and induce expression of the BAG1 and LDH2 bradyzoite marker genes in type II ME49 tachyzoites (6). These findings support the idea that translational control mediated by TgIF2 $\alpha$  phosphorylation is an important event in the formation of the latent stage (8). To further explore the role of translational control in *Toxoplasma* infection, we determined the effect of SAL on the replication of ME49 parasites cultured in HFF cells (Fig. 1A). Results show that SAL inhibits tachyzoite replication in a dose-dependent fashion with a 50% effective concentration ( $EC_{50}$ ) of  $\sim 1.5$   $\mu$ M. Vehicle-treated samples contained 8 to 16 parasites per vacuole 32 h postinfection, but only 2 parasites per vacuole were present when 4  $\mu$ M SAL was included in the medium (Fig. 1A).

We also measured the effects of GA on *Toxoplasma* replication *in vitro* since this drug was recently demonstrated to be an eIF2 $\alpha$  dephosphorylation inhibitor (14). Like SAL, GA inhibited tachyzoite replication in a dose-dependent manner, with an  $EC_{50}$  of  $\sim 6$   $\mu$ M (Fig. 1A). Since GA is also an  $\alpha$ 2-adrenergic receptor agonist (13), we examined the effect of the drug clonidine on parasite replication as a control. Like GA, clonidine is an  $\alpha$ 2-adrenergic receptor agonist used in the treatment of hypertension (16). Infected HFFs treated with 15  $\mu$ M clonidine showed no statistically significant difference in the average number of parasites per vacuole compared to results for the vehicle control (Fig. 1A), suggesting that the antiparasitic activities of GA do not involve  $\alpha$ 2-adrenergic receptor agonist activity. SAL and GA also had potent antiparasitic activity against the hypervirulent type I RH strain, with  $EC_{50}$ s similar to those seen for the ME49 strain

(Fig. 1B). As observed for the ME49 strain, we found that clonidine had no antiproliferative activity against RH strain tachyzoites.

**SAL and GA induce tachyzoite conversion to latent bradyzoites *in vitro*.** Since SAL and GA treatment of tachyzoites induced expression of bradyzoite-specific marker genes (6) (see Fig. SA2 in the supplemental material), we next addressed if the antiproliferative effect of SAL and GA was due to conversion of tachyzoites to latent bradyzoites. Incubation of intracellular tachyzoites in alkaline media is a well-established method to induce formation of bradyzoite tissue cysts *in vitro*, which can be visualized by IFA using the *Dolichos biflorus* lectin that binds cyst wall protein (17). In dose-dependent fashion, both SAL and GA induced bradyzoite formation *in vitro*, with 4.0 and 10.0  $\mu$ M concentrations yielding roughly the same number of cysts at day 7 as the alkaline-pH control, respectively (Fig. 2). Interestingly, tachyzoites exposed to 8.0  $\mu$ M SAL for 7 days exhibited unusual morphology, with lectin-stained protein failing to reach the cyst wall (see Fig. SA1 in the supplemental material). These abnormal parasites also appeared to be limited to one per vacuole. These data suggest that SAL is lethal to parasites at doses exceeding 8.0  $\mu$ M, but sublethal doses induce bradyzoite conversion, as found for GA. Higher doses of GA may also be lethal for *Toxoplasma*, but doses in excess of 25  $\mu$ M begin to stress the host cell monolayer, leading to its detachment from the flask.

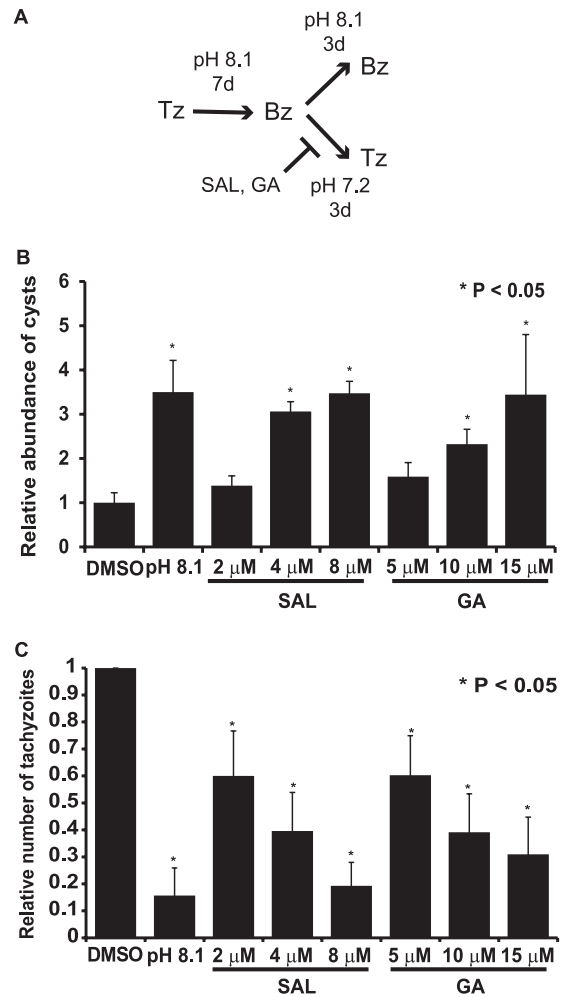
**SAL and GA inhibit reactivation of latent bradyzoites *in vitro*.** We previously reported that latent bradyzoites have sustained TgIF2 $\alpha$  phosphorylation (6). If TgIF2 $\alpha$  phosphorylation is critical to form and maintain the cyst form, one would predict that inhibitors of TgIF2 $\alpha$  dephosphorylation would attenuate the reactivation of infection, i.e., block reconversion of bradyzoites into proliferative tachyzoites. The reactivation of infection can be examined *in vitro* since removal of the stress used to induce bradyzoite development allows rapid reconversion to tachyzoites. As described above, we induced formation of bradyzoite tissue cysts using alkaline medium for 7 days. The bradyzoite-containing monolayers were then washed and incubated in fresh medium of



**FIG 2** SAL and GA induce tachyzoite conversion to latent bradyzoites *in vitro*. HFF cells infected with ME49 strain parasites were cultured with the indicated concentration of SAL, GA, or vehicle (DMSO) for 7 days. As a control, infected HFFs were grown in alkaline medium (pH 8.1) for 7 days. The frequency of cyst formation was assessed by counting *Dolichos* lectin-stained cysts in at least 25 random fields on day 7. The graph shows averages for an experiment performed in triplicate and normalized to the number of cysts present in the DMSO control sample. An independent experiment showed similar results. Error bars indicate standard deviations, and asterisks indicate a statistically significant difference relative to results for the vehicle control ( $P < 0.05$ , two-tailed unpaired  $t$  test).

normal physiological pH (pH 7.2). As a control, a parallel sample of bradyzoites was retained in alkaline medium to prevent reactivation (Fig. 3A). As expected, the relative number of cysts was approximately 3.5-fold higher in the samples kept in alkaline medium than in the samples that had alkaline medium replaced with normal medium of pH 7.2 (Fig. 3B). However, in the presence of at least 4  $\mu$ M SAL or 10  $\mu$ M GA, the reactivation of latent bradyzoites was significantly inhibited, with the relative number of cysts being approximately 2.5- to 3.0-fold higher than that in the normal medium supplemented with DMSO (Fig. 3B). Samples with higher concentrations of SAL or GA contained as many bradyzoite cysts as the nonreactivated samples maintained in alkaline pH (Fig. 3B). We also determined the number of tachyzoites in the infected cell culture supernatant following removal of the alkaline medium as an independent measure of the reactivation of infection. Increasing concentrations of SAL or GA were found to reduce the number of tachyzoites in the reactivated cultures (Fig. 3C). These results strongly suggest that SAL and GA can inhibit the reactivation of latent bradyzoites upon stress removal *in vitro*.

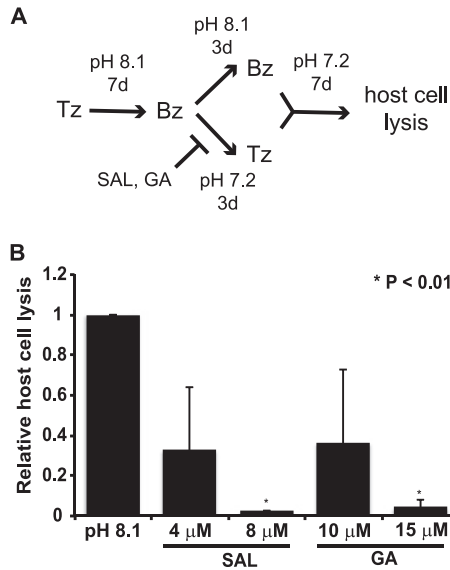
To analyze whether SAL/GA-treated cysts suffer a permanent inability to reactivate, bradyzoite cysts subjected to SAL or GA for 3 days were allowed to recover in normal medium without the drug. The experimental setup is shown in Fig. 4A. After 7 days with no drugs or stress, the infected monolayers were fixed, and relative host cell lysis was determined as a measure of reactivated infection. As a standard, we measured the reactivation of bradyzoite cysts that were maintained on alkaline medium without drug for 3 days and then cultured in normal medium for 7 days. Reactivation of infection was detected in cultures exposed to 4  $\mu$ M SAL or 10  $\mu$ M GA (Fig. 4B). However, significantly less reactivation was detected in cultures treated with 8  $\mu$ M SAL or 15  $\mu$ M GA (Fig. 4B). These data further support the idea that SAL and GA prevent reconversion of bradyzoites into replicating tachyzoites and suggest that the failure to reactivate infection can persist for a time even after the drug is removed from culture. Furthermore, it is



**FIG 3** SAL and GA impede reactivation of latent bradyzoites *in vitro*. (A) The diagram outlines the experimental setup. ME49 tachyzoites (Tz) were differentiated into bradyzoite cysts (Bz) using alkaline (pH 8.1) medium for 7 days. The alkaline stress medium was then removed and replaced with normal medium (pH 7.2) containing indicated concentrations of SAL, GA, or vehicle (DMSO) for 3 days. Alkaline medium was retained in some samples as a positive control to prevent reactivation. Reactivation was assessed by counting *Dolichos* lectin-stained cysts in at least 25 random fields (B) or by counting reactivated tachyzoites present in culture medium (C). (B) The histogram shows the results from a representative experiment performed in triplicate and normalized to the number of cysts counted in the vehicle control. This experiment was repeated a total of three times, each with similar results. (C) The average for three independent experiments was normalized to the number of tachyzoites present in the vehicle control. In panels A and B, error bars indicate standard deviations and asterisks indicate a statistically significant difference relative to results for the vehicle control ( $P < 0.05$ , two-tailed unpaired  $t$  test).

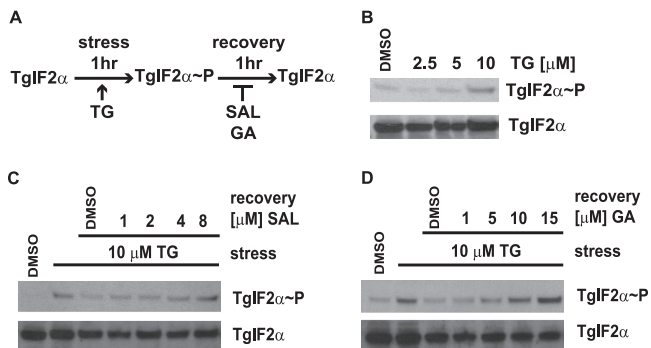
possible that the higher concentrations of SAL and GA are lethal to bradyzoites.

**SAL and GA inhibit dephosphorylation of TgIF2 $\alpha$ .** SAL and GA are recognized inhibitors of eIF2 $\alpha$  dephosphorylation in mammalian cells (12, 14). An assay that takes advantage of the fact that TgIF2 $\alpha$  becomes phosphorylated in purified tachyzoites exposed to pharmacological agents inducing endoplasmic reticulum (ER) stress has been established (6). Removal of the stress treatment causes rapid dephosphorylation of TgIF2 $\alpha$ , but the inclusion of SAL can prevent dephosphorylation of TgIF2 $\alpha$ . We tested

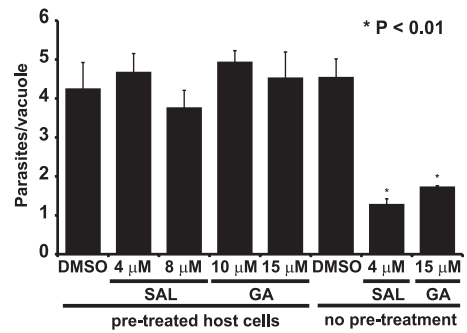


**FIG 4** SAL- and GA-treated tissue cysts are defective for reactivation. After cysts were generated for 7 days, the alkaline stress medium was removed and replaced with normal medium containing the indicated concentrations of SAL or GA for an additional 3 days. Alkaline medium (pH 8.1) was retained in some samples as a positive control to prevent reactivation. Following the incubation in normal medium without SAL or GA for another 7 days, the extent of reactivation of tachyzoite infection was assessed by determining the degree of host cell lysis relative to that for the alkaline control using an Alpha Innotech imager. (A) The diagram shows a time line of the experimental setup. (B) The histogram shows the results from two experiments, each performed in triplicate. Error bars indicate standard deviations, and asterisks indicate a statistically significant difference relative to results for the alkaline control ( $P < 0.05$ , two-tailed unpaired  $t$  test).

if SAL and GA could inhibit dephosphorylation of TgIF2 $\alpha$  in response to another ER stress agent, thapsigargin. Purified tachyzoites treated with thapsigargin for 60 min exhibited a concentration-dependent induction of TgIF2 $\alpha$  phosphorylation (Fig. 5A and B). One hour after removing the stress, we observed nearly complete dephosphorylation of TgIF2 $\alpha$  (Fig. 5C and D). However, SAL and GA inhibited this dephosphorylation of TgIF2 $\alpha$  in a dose-dependent manner (Fig. 5C and D). These results suggest



**FIG 5** SAL and GA inhibit TgIF2 $\alpha$  dephosphorylation. (A) Schematic diagram of experimental design. (B) Purified ME49 tachyzoites were treated with the indicated concentrations of thapsigargin (TG) or vehicle (DMSO) for 60 min. Following exposure to 10  $\mu$ M TG for 60 min, tachyzoites were allowed to recover in the presence of vehicle (DMSO) or the indicated concentrations of SAL (C) or GA (D). Equal amounts of protein lysate were separated by SDS-PAGE, followed by immunoblotting using antibody that recognizes total TgIF2 $\alpha$  or phosphorylated TgIF2 $\alpha$  (TgIF2 $\alpha$ -P).

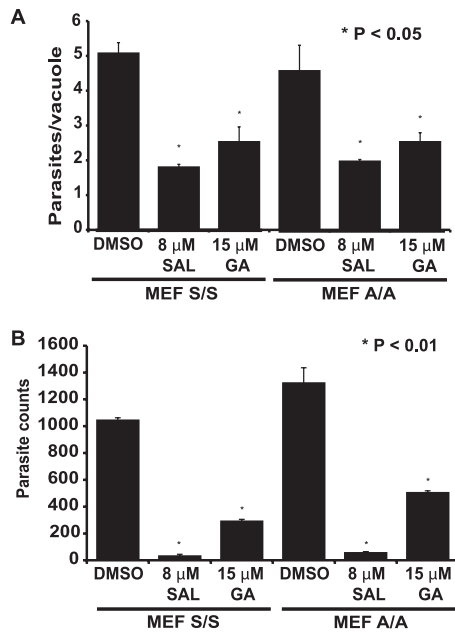


**FIG 6** Pretreatment of HFF cells with SAL or GA does not affect tachyzoite infection. Confluent HFF monolayers were treated with the indicated concentrations of SAL, GA, or vehicle (DMSO) for 24 h ("pre-treated host cells"). Following two washing steps in medium, the pretreated HFF cells were infected with ME49 tachyzoites. At 24 h postinfection, the number of parasites per vacuole was determined for at least 50 random vacuoles. As a control, replication in the presence of the indicated concentration of SAL, GA, or vehicle (DMSO) was also assessed ("no pre-treatment"). Histograms show averages from a triplicate experiment. Error bars indicate standard deviations, and asterisks indicate a statistically significant difference from results for the vehicle control ( $P < 0.01$ , two-tailed unpaired  $t$  test).

that both SAL and GA inhibit TgIF2 $\alpha$  dephosphorylation in stressed tachyzoites.

**Antiparasitic activities of SAL and GA do not involve host eIF2 $\alpha$ .** While the previous experiment indicates that SAL and GA are capable of adversely affecting the dephosphorylation of TgIF2 $\alpha$ , the antiparasitic action of the drugs may also involve inhibition of host eIF2 $\alpha$  dephosphorylation. We addressed the possibility that SAL or GA adversely affects the parasites by acting through the host cell in two different ways. First, we reasoned that if SAL and GA function through the host cells, treatment with either drug prior to infection may affect parasite replication. Such effects of drug preconditioning were reported by Radke et al., who demonstrated that pretreatment of HFF host cells with compound 1 (a trisubstituted pyrrole and bradyzoite inducer) prior to infection with tachyzoites reduced parasite proliferation by promoting bradyzoite formation (18). We therefore pretreated HFF monolayers with increasing concentrations of SAL, GA, or vehicle for 24 h prior to infection and then counted the number of parasites per vacuole 24 h postinfection. Preconditioning host cells with either SAL or GA had no significant effect on parasite replication (Fig. 6), suggesting that these drugs do not modify host cells in a way that makes them less hospitable for infection.

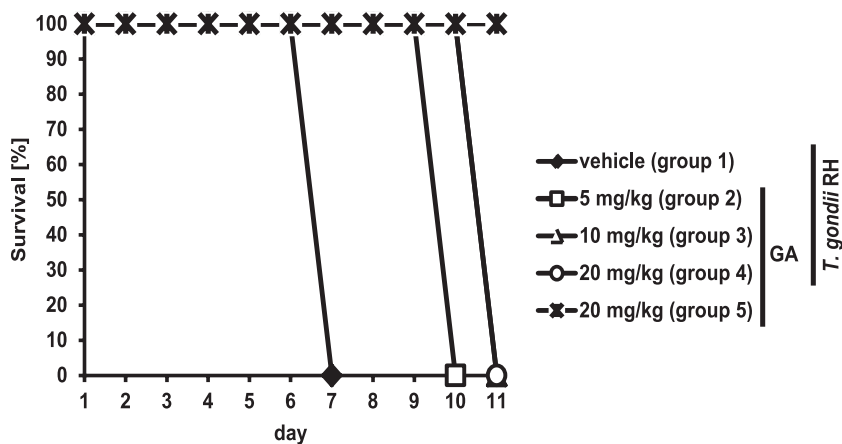
As an alternative way to test if the antiparasitic activities of SAL and GA involved effects on host eIF2 $\alpha$ , we infected a mouse embryonic fibroblast (MEF) cell line engineered to express a nonphosphorylatable version of eIF2 $\alpha$  (eIF2 $\alpha$ -S51A), which would negate the effects of either drug on host translational control (19). Wild-type (S/S) and mutant (A/A) MEF cells were infected with ME49 tachyzoites in the presence or absence of SAL or GA, and the number of parasites per vacuole was determined 24 h postinfection. The antiparasitic effects of SAL and GA were identical in both S/S and A/A MEF cells, suggesting that host cell eIF2 $\alpha$  is not a contributor (Fig. 7A). Due to difficulties in visualizing parasites within MEF cells at 48 h, we used quantitative PCR and primers specific for the *Toxoplasma* B1 gene to monitor infection levels. Once again, tachyzoite replication was sharply reduced in the presence of SAL or GA in both S/S and A/A cells (Fig. 7B). These results suggest that the mechanism of action of



**FIG 7** SAL and GA reduce *Toxoplasma* replication regardless of host cell eIF2 $\alpha$  phosphorylation. Tachyzoites were allowed to infect MEFs expressing wild-type (S/S) or nonphosphorylatable (A/A) eIF2 $\alpha$  in culture medium supplemented with the indicated concentration of SAL, GA, or vehicle (DMSO). (A) At 24 h postinfection, parasite replication was assessed by counting tachyzoites visualized by IFA through labeling with an Alexa 488-conjugated anti-SAG1 antibody in at least 50 random vacuoles. The graph shows the averages for an experiment performed in triplicate. Error bars indicate standard deviations, and asterisks indicate a statistically significant difference relative to the vehicle control ( $P < 0.05$ , two-tailed unpaired  $t$  test). (B) At 48 h postinfection, parasite replication was assessed using the PCR-based B1 assay. The histograms show the averages for a representative experiment performed in triplicate. The experiment was repeated three independent times with similar results. Error bars indicate standard deviations, and asterisks indicate a statistically significant difference relative to results for the vehicle control ( $P < 0.01$ , two-tailed unpaired  $t$  test).

SAL and GA against *Toxoplasma* infection does not involve host cell eIF2 $\alpha$  phosphorylation.

**GA extends survival of mice acutely infected with *Toxoplasma*.** The data presented suggest that GA and SAL adversely



**FIG 8** GA extends the survival of mice acutely infected with *Toxoplasma*. Mice in groups 1 to 4 were infected with a lethal dose of 100 *Toxoplasma* tachyzoites (RH strain) and treated with vehicle (group 1) or the indicated dose of GA (groups 2 to 4). To exclude adverse effects of GA on survival, a noninfected group receiving the same dose of GA as group 4 was included (group 5). Survival of each treatment group is presented for the time course of the experiment.

affect TgIF2 $\alpha$  dephosphorylation, reduce *Toxoplasma* replication, and promote bradyzoite formation and stability *in vitro*. To address whether GA affects *Toxoplasma* infection *in vivo*, BALB/c mice were infected with a lethal dose of 100 RH strain tachyzoites and treated with GA or vehicle every other day throughout the course of infection. In this model of acute infection, the hyper-virulent RH parasites cause a moribund state 6 to 7 days postinfection (10). As expected, all infected mice treated with vehicle alone were moribund by day 7. In contrast, infected mice treated with GA did not reach a moribund state until up to 11 days. Mice treated with a total dosage of 5 mg/kg GA every other day (group 2) survived 3 days longer, and mice treated with a total dosage of 10 or 20 mg/kg every other day (groups 2 and 4, respectively) survived 4 additional days compared to the infected mice receiving vehicle (Fig. 8). The course of infection and the onset were similar for all mice within each group. At day 11, all uninfected mice treated with the highest dose of GA appeared normal, suggesting that this dosing regimen does not affect the viability of the mice. Tachyzoites were found in the peritoneal fluid of all infected mice, suggesting that mice succumbed to infection rather than to adverse effects of drug treatment.

## DISCUSSION

During the course of *Toxoplasma* infection, proliferating tachyzoites evade the immune system by converting into latent bradyzoite cysts, which can persist in host tissues for life. If the immune system of the host becomes compromised, bradyzoites can reconvert into destructive tachyzoites, leading to dangerous reactivation of parasite infection (3). Current treatments for toxoplasmosis produce serious adverse effects and are thought to primarily target only tachyzoites, underscoring the need for safer treatment and prophylactic options (1).

This study showed that the drugs SAL and GA significantly slow the replication of *Toxoplasma* tachyzoites in cultured HFF host cells, with EC<sub>50</sub>s of 1.5 and 6  $\mu$ M, respectively (Fig. 1). Furthermore, since slowed parasite proliferation is a precursor for development into the latent bradyzoite cyst stage (20), we determined if SAL- or GA-treated tachyzoites were expressing cyst wall proteins, using FITC-conjugated *Dolichos* lectin staining. Our findings indicate that SAL and GA are potent promoters of

tachyzoite-to-bradyzoite conversion (Fig. 2). Bradyzoite cysts treated with SAL or GA failed to reactivate into tachyzoites upon removal of the induction stress (Fig. 3B and C). Drug-treated cysts appeared to sustain an irreversible defect that rendered them incapable of reconvert to tachyzoites even when drug was removed (Fig. 4). While this could be due to residual drug retained in the cells, it opens the exciting possibility that high levels of TgIF2 $\alpha$  phosphorylation in response to SAL and GA may be lethal to bradyzoites.

GA offers several significant advantages over SAL in terms of *in vivo* usage. First, GA is a safe, FDA-approved drug already in clinical use for hypertension. Second, GA crosses the blood-brain barrier, which is necessary to target latent bradyzoites (or reactivated tachyzoites) in the brain. These features, considered with the ability of GA to slow parasite replication *in vitro*, prompted our examination of GA in the mouse model of acute toxoplasmosis infection. Mice infected with the RH strain reached the moribund state after 6 days, as expected, but mice treated with GA survived at least 3 days longer (Fig. 8). Prolonged survival of mice infected with the hypervirulent RH strain, which has a 50% lethal dose (LD<sub>50</sub>) of ~1 and does not readily form latent cysts, is promising. It is also noteworthy that the GA-treated mice showed little sign of toxicity at the dosages used here. Optimization of the dose of GA or combination with another antiparasitic may increase survival time. These encouraging results highlight the importance of characterizing the target of GA to develop more potent inhibitors. It is noted that GA may exert greater activity against a cyst-generating strain or be effective at blocking reactivation of infection *in vivo*, and therefore, future studies are needed to examine the efficacy of GA against chronic toxoplasmosis in the mouse model. High-throughput drug screening has found that GA exerts antiproliferative activity against various *Plasmodium falciparum* strains at concentrations between 4 and 11  $\mu$ M (PubChem BioAssay; <http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?cid=5702062>; see also reference 21), suggesting that GA may have activity against a number of parasitic protozoa.

GA was found to bind to GADD34 (PPP1R15A), a regulatory protein of the PP1 dephosphorylation complex, thereby enhancing phosphorylated eIF2 $\alpha$  (eIF2 $\alpha$ ~P) levels and providing cytoprotection during cellular stress (14). It is noted that GA binds and interferes only with GADD34-mediated dephosphorylation of eIF2 $\alpha$ , whereas SAL also associates with and blocks the function of CReP (PPP1R15B), which facilitates basal eIF2 $\alpha$  dephosphorylation (12). Therefore, GA is thought to attenuate recovery of translation following stress, while SAL can trigger enhanced eIF2 $\alpha$ ~P and repressed protein synthesis in the absence of additional stress. These mechanisms of action may explain why SAL is more potent against *Toxoplasma* than GA, with concentrations of 8.0  $\mu$ M or more appearing to be lethal.

Given that *Toxoplasma* is an obligate intracellular parasite, we considered that the antiparasitic effects of SAL and GA might occur indirectly through interference with host cell eIF2 $\alpha$  dephosphorylation. We addressed this issue with a number of independent approaches. First, as we have shown previously, SAL has a direct effect on TgIF2 $\alpha$  phosphorylation independent of host cells (6). SAL has also been shown to increase RNA granule formation in extracellular *Toxoplasma*, which occurs during eIF2 $\alpha$  phosphorylation, and SAL can enhance the levels of *Plasmodium* eIF2 $\alpha$  phosphorylation (22, 23). Here we have further demonstrated this interaction by using extracellular tachyzoites treated with thapsigargin, which potently induces phosphorylation of eIF2 $\alpha$ . Upon

removal of the thapsigargin, TgIF2 $\alpha$  is rapidly dephosphorylated, but inclusion of SAL or GA directly prevents the dephosphorylation of TgIF2 $\alpha$  (Fig. 5). Second, we preconditioned the HFF host cells with SAL or GA prior to infection with *Toxoplasma*, but the drugs did not alter the host cell in a way that was detrimental to parasite invasion or replication (Fig. 6). Finally, SAL and GA reduced *Toxoplasma* infection even when we used host cells that were genetically engineered to be incapable of phosphorylating eIF2 $\alpha$  (Fig. 7). Considered together, the data suggest that the mechanisms of action of SAL and GA do not involve host cell eIF2 $\alpha$  phosphorylation. However, it is possible that SAL or GA can also exert their effects by one or more mechanisms that are independent of eIF2 $\alpha$  phosphorylation. These independent mechanisms may involve either host or parasite stress response pathways.

Despite clear evidence that SAL and GA can directly prevent TgIF2 $\alpha$  dephosphorylation, how this may occur in the parasite requires further investigation. Bioinformatics analysis of the ToxoDB showed the presence of an unequivocal PP1 homologue (TGME49\_110700) but did not identify clear orthologues of PP1 regulatory proteins GADD34 or CReP. This could simply be a matter of primary sequence divergence in *Toxoplasma*; i.e., functional equivalents of these PP1 regulatory proteins may exist and serve as the target of SAL and/or GA. However, we cannot exclude the potential roles of additional targets in *Toxoplasma* at this time.

Recent studies in other parasites, such as *Plasmodium*, *Leishmania*, and *Trypanosoma*, have supported our earlier findings that eIF2 $\alpha$  phosphorylation and translational control play a critical role in parasite development (11, 22, 24–26). The findings presented here further underscore the significance of translational control during parasite proliferation and development into latent stages. Given its proven safety record, our discovery of the antiparasitic effects of GA in particular could rapidly lead to a new prophylactic treatment to prevent reactivated toxoplasmosis and perhaps other protozoal infections as well.

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