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A High-Throughput Amenable Dual Luciferase System for Measuring *Toxoplasma gondii* Bradyzoite Viability after Drug Treatment

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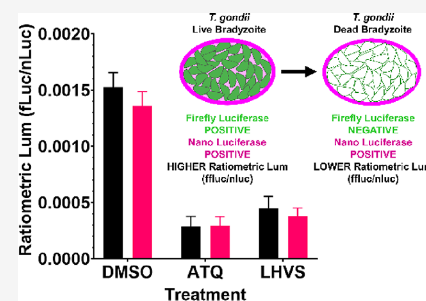
ABSTRACT: It is estimated that more than 2 billion people are chronically infected with the intracellular protozoan parasite *Toxoplasma gondii* (*T. gondii*). Despite this, there is currently no vaccine to prevent infection in humans, and there is no recognized curative treatment to clear tissue cysts. A major hurdle for identifying effective drug candidates against chronic-stage cysts has been the low throughput of existing *in vitro* assays for testing the survival of bradyzoites. We have developed a luciferase-based platform for specifically determining bradyzoite survival within *in vitro* cysts in a 96-well plate format. We engineered a cystogenic type II *T. gondii* PruΔ*ku80*Δ*hxgpr* strain for stage-specific expression of firefly luciferase in the cytosol of bradyzoites and nanoluciferase for secretion into the lumen of the cyst (DuaLuc strain). Using this DuaLuc strain, we found that the ratio of firefly luciferase to nanoluciferase decreased upon treatment with atovaquone or LHVS, two compounds that are known to compromise bradyzoite viability. The 96-well format allowed us to test several additional compounds and generate dose−response curves for calculation of EC_{50} values indicating relative effectiveness of a compound. Accordingly, this DuaLuc system should be suitable for screening libraries of diverse compounds and defining the potency of hits or other compounds with a putative antibradyzoite activity.



■ INTRODUCTION

Toxoplasma gondii is the causal agent of toxoplasmosis, a disease that can lead to serious health outcomes.[1−3] For example, congenital toxoplasmosis can result in microcephaly, hepatosplenomegaly, or chorioretinitis in the developing fetus or even miscarriage.[4−6] Ocular toxoplasmosis can lead to blindness, and infection in immunocompromised individuals can lead to death.[3,7,8] During the acute stage when rapidly dividing tachyzoites predominate, the parasite disseminates throughout host tissues and is commonly associated with tissues that have low host cell turnover or immune-privileged sites, permitting chronic infection.[9] Infections in heart tissues have been associated with myocarditis,[3,10] and the parasite has also been correlated with tumors in the lungs of human cancer patients.[11] The chronic infection, which is manifested by slowly growing bradyzoites contained within intracellular tissue cysts, is typically associated with the brain and muscle and has been associated with a number of psychological and behavioral conditions, including schizophrenia.[12] Because of the high infection rate and the severity of potential disease outcomes, toxoplasmosis is designated as the third most important foodborne disease within Europe by the European Food Standards Agency and a highly important foodborne disease worldwide by the World Health Organization, based on Disability Adjusted Life Years (DALYs).[13,14] Despite this, there is no officially recognized treatment against chronic infection.

Drug development against chronic toxoplasmosis has been restrained by limited assay development for determining chronic stage bradyzoite viability. Among the few assays that have been developed, Araujo *et al.* treated *ex vivo* cysts and measured viability by staining with acridine orange and ethidium bromide or by reinfection of mice.[15] Although the use of cysts from infected mice is an asset, such cysts were manipulated by forced liberation from host cells. Also, the staining method was only moderately quantitative, and mouse infection experiments are low-throughput and expensive. Murata *et al.* used parasites expressing bradyzoite-specific firefly luciferase to test the efficacy of two compounds.[16] However, the assay did not distinguish effects of treatment on bradyzoite growth versus those on bradyzoite viability. Also, treatment was administered to 3-day differentiated cultures, which likely contain parasites that are still in the process of stage conversion. Di Cristina *et al.* combined quantitative PCR and plaque assay to measure the viability of *in vitro* bradyzoites,

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but the assay is low-throughput and lengthy because of the 10- to 15-day plaquing period.[17]

Here, we report the development of a dual luciferase-based (DuaLuc) assay for measuring *in vitro* bradyzoite survival in 96-well plates. In this system, host cells are seeded with tachyzoites of a dual firefly luciferase (fLuc)- and modified nanoluciferase (nLuc)-expressing parasite strain. Parasites are converted to bradyzoites over seven days, whereby stage-specific expression of both luciferases occurs (Figure 1A). In live bradyzoites, nLuc is directed to the parasite extracellular cyst space (matrix and cyst wall), while fLuc is expressed in the parasite cytosol. fLuc is lost upon bradyzoite death, while nLuc remains fixed, allowing a ratiometric readout of the luciferase activity to be used as a measurement of bradyzoite viability (Figure 1B). Furthermore, we show that this system can be applied to determine the efficacy of compounds against the chronic infection life stage of the parasite, for which no approved drug is currently commercially available.

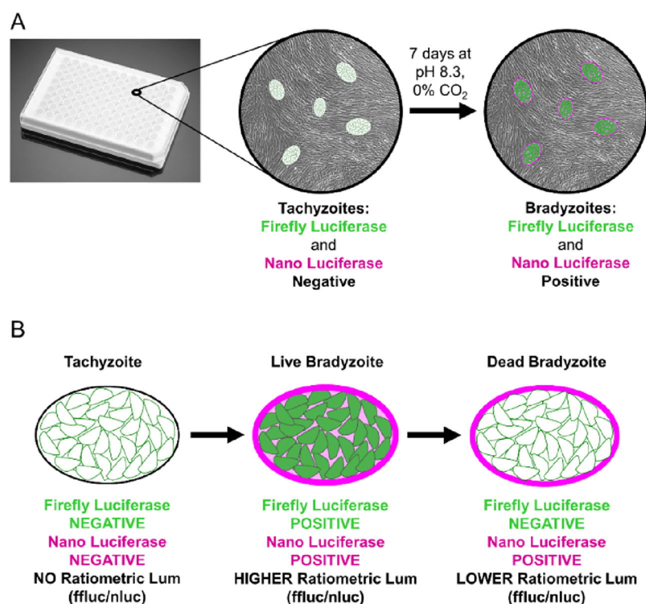

Figure 1. Overview of the DuaLuc system. (A) Schematic of the bradyzoite-specific dual luciferase expression system. Expressions of firefly luciferase fLuc and nLuc are driven by the bradyzoite-specific *BAG1* or *LDH2* promoter, respectively, and therefore, luciferase expression will only take place in differentiated parasites (*e.g.*, in response to alkaline stress *in vitro*). (B) fLuc is expressed in the parasite cytosol and is the variable marker to determine live bradyzoites. nLuc is directed to the cyst wall where it remains fixed upon bradyzoite death and is therefore the static normalization marker.

EXPERIMENTAL SECTION

Cell Lines. Throughout this investigation, human foreskin fibroblast (HFF) cells were used to propagate parasite strains in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (w/v) cosmic calf serum (CCS).

The *T. gondii* strain PruΔ*ku80*Δ*hxgpr*[18] was used as the background strain in this study and was modified to generate DuaLuc-expressing strains, as described below.

Bradyzoite Differentiation. Bradyzoite differentiation was performed as described by Smith *et al.*[19] For all bradyzoite conversion, tachyzoites were mechanically lysed by scraping infected HFF monolayers that were then passed sequentially through 20G and 23G syringes and a 3 μm filter. Filtered parasites were then counted and allowed to infect fresh monolayers of HFF cells for 24 h. Bradyzoite differentiation was induced using alkaline pH medium and ambient CO_2.[20−23] After 24 h, DMEM media was replaced for an alkaline differentiation media (RPMI without $NaHCO_3$, 50 mM HEPES, pen/strep, and 1% FBS, pH 8.25). Differentiation media was replaced daily.

Immunofluorescence. HFF cell monolayers were grown on coverslips and infected with tachyzoites. They were either fixed 48 h postinfection as tachyzoites or following 7 days of bradyzoite differentiation, as described above. Infected monolayers on coverslips were fixed for 30 min with 4% (w/v) paraformaldehyde in PBS. Permeabilization was carried out by incubating the fixed samples in 0.1% Triton X-100 in PBS for 10 min. Samples were blocked with 10% cosmic calf serum (CCS) in PBS. Rat anti-HA (Sigma-Aldrich, cat. no. 11867423001) (1:500) and rabbit antimyc (Cell Signaling Technologies, cat. no. 2278S) (1:500) primary antibodies in wash buffer (WB; 1% CCS in PBS) were subsequently applied to samples and left for 1 h at RT. Samples were washed three times for 5 min with WB. Secondary antibodies were goat antirat Alexa Fluor 594 (Invitrogen, cat. no. A11007) (1:1000) and goat antirabbit Alexa Fluor 350 (Invitrogen, cat. no. A-11046) (11000). Fluorescein *Dolichos biflorus* (*D. biflorus*) agglutinin was used at 1:400 (Vector Laboratories, cat. nos. FL-1031 and B-1035). Samples were incubated in secondary antibodies and fluorescein *D. biflorus* agglutinin simultaneously (in WB) for 1 h followed by three washes with WB. Coverslips were mounted with mowiol. Images were taken on a Zeiss Axio Observer Z1 inverted microscope at 100× and analyzed using Zen 3.0 blue edition software.

Immunofluorescence confocal microscopy was performed to see if the myc-tagged nLuc protein is associated with the cyst wall of *in vitro* bradyzoites. Following seven days of *in vitro* bradyzoite differentiation, infected HFF cell monolayers were fixed with 4% (w/v) PFA and made permeable with 0.1% Triton X-100. Samples were labeled with mouse α-myc antibodies at a dilution of 1:100, as well as labeled with biotinylated Dolichos lectin to label the cyst wall. Secondary goat α-mouse 594 was used to indirectly label the Myc antigen, while streptavidin Alexa Fluor 350 was used at 1:1000 dilution to indirectly fluorescently label the cyst wall. Images were captured using a Nikon Yokogawa spinning disk microscope.

Dual Luciferase Assays. Confluent HFF cell monolayers on white-walled, flat, clear-bottom 96-well tissue culture plates (Corning, cat. no. 3610) were infected with *T. gondii* tachyzoites. After 24 h, tachyzoites were converted to bradyzoites as described above. Wells were monitored daily to ensure that parasites remained intracellular and that the host cell monolayer was intact. At the end of the culture period, media was removed, and the bottom of the plate was covered with a white adhesive sticker; the fLuc and nLuc activity was measured at set time points using a Nano-Glo Dual-Luciferase Reporter Assay System (Promega, cat. no. N1620), following the manufacturer's instructions (outlined in Figure 3A, detailed protocol in the Supporting Information file). The luciferase activity (lum) was measured using a Bio-Tek Synergy HT microplate reader.

Validation of the Dual Luciferase System for Determining Bradyzoite Viability. Confluent HFF cell monolayers on white-walled, flat, clear-bottom 96-well tissue

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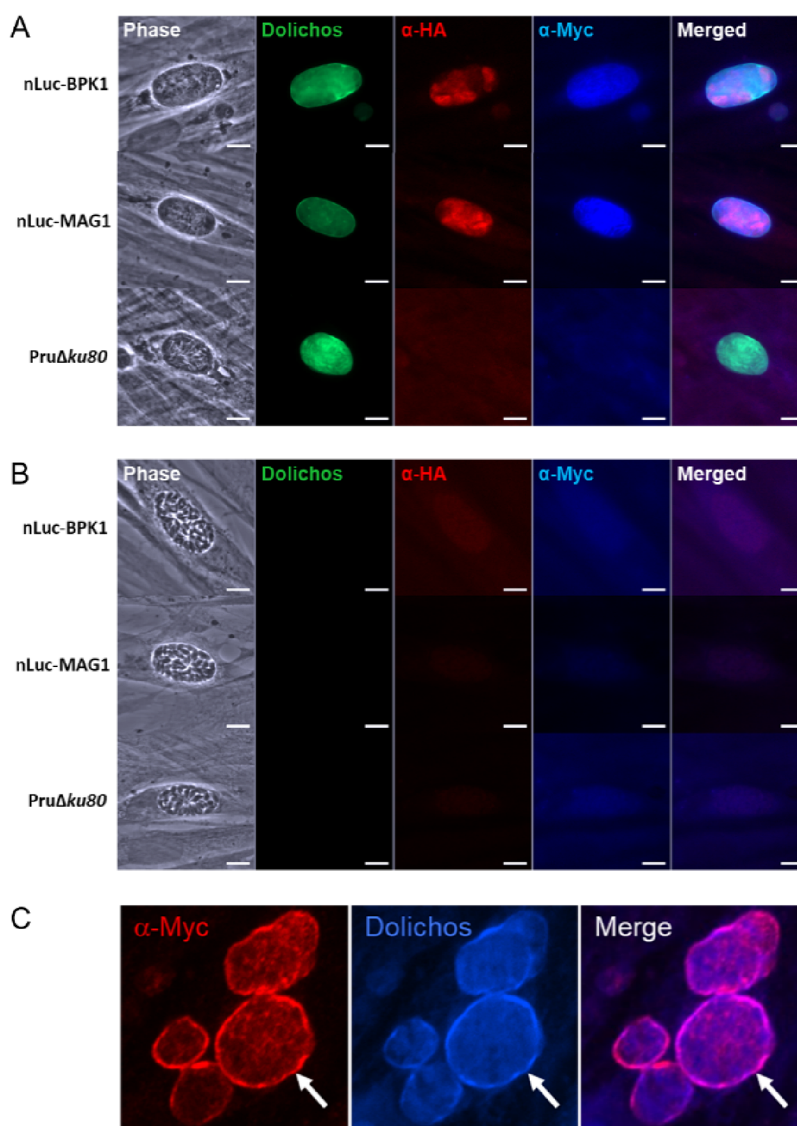

Figure 2. Development of bradyzoite-specific DuaLuc-expressing parasites. (A) Expression of HA-tagged fLuc and myc-tagged nLuc confirmed in modified parasites and not in the parental PruΔ*ku80*Δ*hxgpr* strain. (B) fLuc and nLuc are not expressed in Dolichos-negative infected cells. Scale bar = 10 μm. (C) Immunofluorescence confocal microscopy demonstrating that the myc-tagged nLuc protein is trafficked to the cyst wall of *in vitro* bradyzoites. White arrows indicate colocalization of the myc antigen and cyst wall lectin at the boundary of an *in vitro* bradyzoite cyst.

culture plates were infected with 1×10^2 *T. gondii* tachyzoites, which were then converted to bradyzoites over the course of 7 days, as described above. After 7 days of bradyzoite differentiation, wells were treated with either 0.1% DMSO (vehicle control), 20 μM atovaquone (ATQ), or 5 μM morpholine-leucine homophenylalanine vinyl sulfone (LHVS) in differentiation media. Treatments were replaced daily for up to 14 days. Following the treatment period, media was removed, and the base of the plate was covered with a white adhesive sticker. The fLuc and nLuc activity was then measured as described above. Wells challenged with parental PruΔ*ku80* tachyzoites lacking fLuc and nLuc expression were used to calculate plate background luminescence.

To determine ratiometric luminescence (ratiometric lum), the fLuc activity (lum) was divided by the nLuc activity (lum), based on our reasoning that the cytosolic fLuc activity would be variable and lost in dead/dying bradyzoites, while the secreted nLuc activity would be static and remain fixed in the event of bradyzoite death. nLuc is therefore the normalizer for the total parasite load (dead or alive), while fLuc is variable and enumerates only the live parasite load.

Quantitative PCR-plaque assays were performed as described previously by us.[17,19] Following *in vitro* cyst formation, parasites were treated with 20 μM ATQ, 5 μM LHVS, or 0.1% DMSO (vehicle control) in differentiation media. Culture media containing the treatment compounds or the vehicle control was refreshed daily for 7 days. Following the treatment period, cysts were liberated by mechanical extrusion, by lifting cells with a cell scraper, and syringing several times through 25G needles. Cysts were exposed to pepsin digest at 37 °C for 30 min (0.026% pepsin in 170 mM NaCl and 60 mM HCl, final concentration). Reactions were stopped with 94 mM Na_2CO_3, samples were centrifuged at 1500*g* for 10 min at RT, and the pepsin-treated parasites were resuspended in 1 mL of DMEM without serum. Parasites were enumerated, and 1500 parasites per well were added to six-well plates containing confluent monolayers of HFFs in D10 media, in triplicate. To allow for the formation of bradyzoite-derived

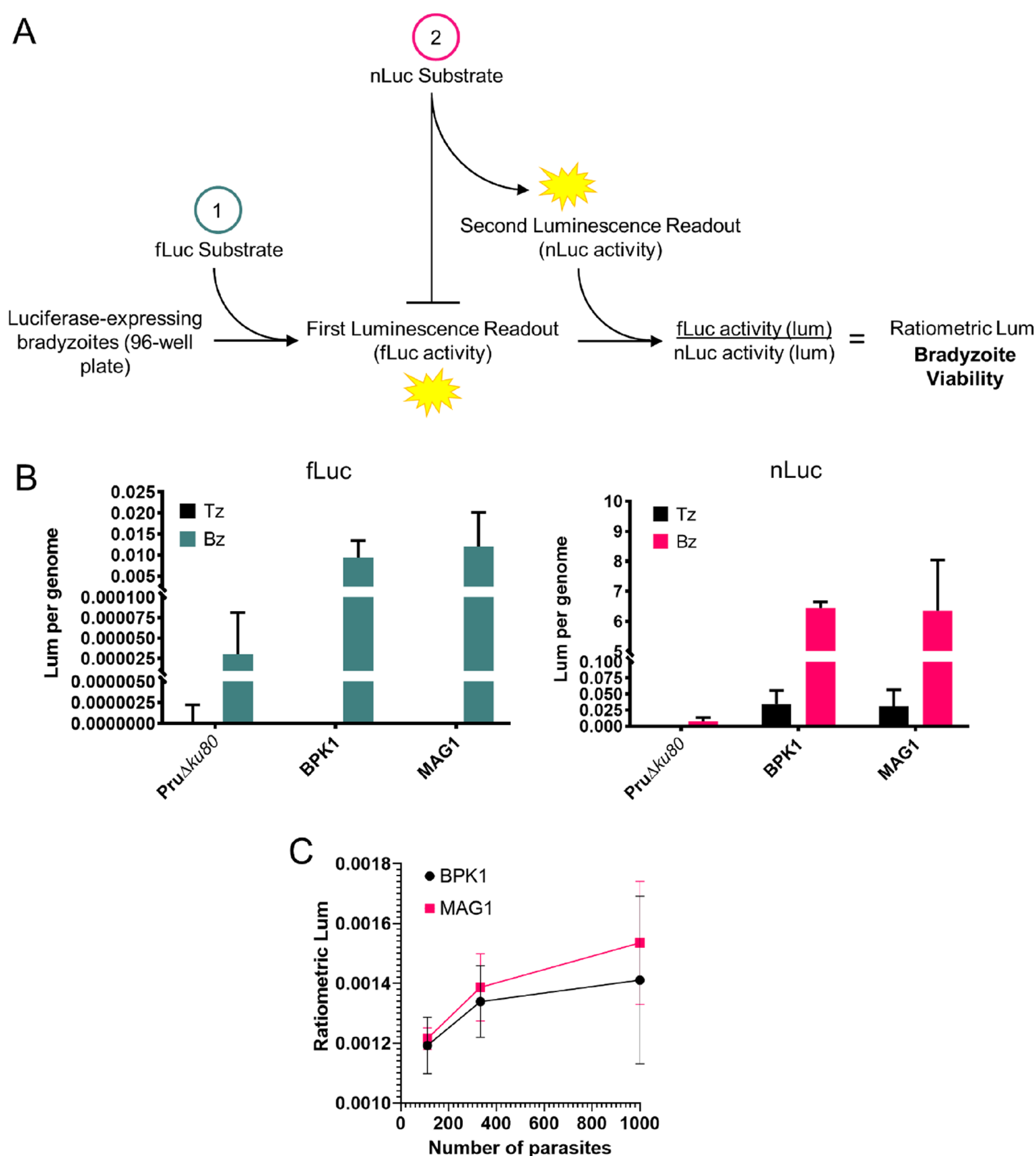

Figure 3. The fLuc and nLuc activity is specific to bradyzoites and not tachyzoites. (A) Diagram of the dual luciferase readout to determine bradyzoite viability. In step 1, the fLuc activity (luminescence, lum) is measured first by addition of an fLuc substrate. In step 2, an fLuc inhibitor and nLuc substrate are added simultaneously, and the nLuc activity (lum) is measured. The fLuc activity (lum) is divided by the nLuc activity (lum) to determine bradyzoite viability. (B) The fLuc and nLuc activity normalized to the parasite genome copy number to determine lum per genome demonstrates that the activity is bradyzoite-specific. (C) The luciferase activity is relative to the number of parasites in the culture, as ratiometric lum (fLuc/nLuc) remains relatively static if wells are seeded with 111, 333, or 1000 tachyzoites at the start of the assay.

plaques, parasites were left to grow undisturbed for 12 days, and then, the number of plaques was enumerated in each well. Five hundred microliters of the initial 1 mL of pepsin-treated parasites was used for genomic DNA purification, performed using a DNeasy Blood and Tissue Kit (Qiagen). Eluted gDNA was quantified by qPCR, in duplicate, using the tubulin primers TUB2.RT.F and TUB2.RT.R.[24] qPCR was performed using a Brilliant II SYBR Green QPCR Master Mix (Agilent) and a Stratagene Mx3000PQ-PCR machine. The number of plaques that formed was then normalized to the calculated number of genomes present in the inoculating sample.

Compound Screen. Since there were no discernable differences in the performance of BPK1-nLuc and MAG1-nLuc strains, we arbitrarily chose to perform subsequent experiments with the BPK1-nLuc strain. Confluent HFF cell monolayers in white-walled, flat, clear-bottom 96-well tissue culture plates were seeded with tachyzoites and differentiated to bradyzoites as described above. After 7 days of bradyzoite differentiation, wells were treated with either 0.1% DMSO (vehicle control) or test compounds in differentiation media. To establish EC_{50} values, test compounds were serially diluted 1:3 from 100 μM to 15 nM and applied to bradyzoite-containing wells. To account for any within-plate well effect, the plate layout was changed between experimental replicates. Media was removed daily and replaced with fresh media containing either the vehicle control only or the test compounds dissolved in DMSO. After 7 days, media was removed, and the luciferase activity was measured as described above. Dose−response curves were generated by performing a $\log_{10}$ transformation of the compound treatment concentration and plotting the relative ratiometric lum activity (normalized against ratiometric lum with 0 μM treatment) at each concentration. A

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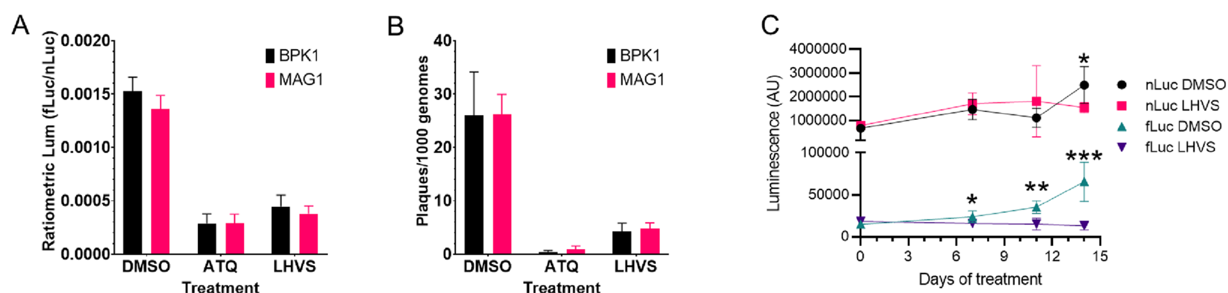

Figure 4. Ratiometric luminescence (lum) decreases following treatment with antibradyzoite compounds. (A) Ratiometric lum (fLuc/nLuc) was found to decrease in bradyzoites of both the BPK1-nLuc and MAG1-nLuc strains following seven days of treatment with 20 μM ATQ and 5 μM LHVS, compared to 0.1% DMSO vehicle-treated control wells (B). This is comparable to the results from qPCR-plaque assays (measured as the number of plaques/1000 parasite genomes) in which bradyzoite cultures underwent the same ATQ, LHVS, and vehicle control treatments (C). A time course assay in which BPK1-nLuc bradyzoites were treated with either 0.1% DMSO or LHVS (5 μM) showed that the differential fLuc activity accounts for the observed decrease in ratiometric lum. *$P < 0.05$, **$P < 0.005$, and ***$P < 0.0005$ (unpaired t-test).

nonlinear regression curve was fitted to the data for each effective compound, and EC_{50} values were determined based on the intercept between $y = 50$ and the curve.

Statistical Analysis. Statistical analysis of ratiometric lum data from the 100 μM compound testing assay using a panel of 12 compounds was performed as follows. Data were analyzed after removing outliers using ROUT with a Q value of 0.1%. Unpaired t-tests without assuming consistent SD were used to test for statistically significant differences in ratiometric lum between 100 and 0 μM compound for each treatment.

RESULTS

Stage-Specific Dual Luciferase Expression in *T. gondii* Bradyzoites. *T. gondii* PruΔ*ku80*Δ*hxgpr* parasites were stably transfected with *bag1*/fLuc-HA for fLuc expression specifically in bradyzoites (Figure 2, Table S1 and Figure S1). This *Bag1*/fLuc-HA strain was then further transfected with either *Ldh2*/BPK1-nLuc-myc or *Ldh2*/MAG1-nLuc-myc for bradyzoite-specific expression of nLuc directed to the cyst matrix and wall (Figure 2 and Table S1).

Stage-specific expression of HA-tagged fLuc and myc-tagged nLuc was confirmed by IFA. Anti-HA and antimyc labeling was only detected in parasites that were also stained positive with the cyst wall marker Dolichos lectin and GFP (already present in the parental strain and expressed under the LDH2 promoter), demonstrating expression of both luciferases specifically in bradyzoites and not tachyzoites (Figure 2). Furthermore, confocal microscopy confirmed the colocalization of BPK1-nLuc-myc with Dolichos lectin at the cyst wall boundary (Figure 2C). Using the assay scheme shown in Figure 3A, we also showed that the luciferase activity is stage-specific based on measuring the activity in 2-day tachyzoite cultures and 7-day bradyzoite cultures normalized to the parasite genome copy number (Figure 3B). BPK1-nLUC and MAG1-nLUC strains exhibited similar levels of the fLuc and nLuc activity based on fLuc and nLuc lum per genome and ratiometric lum (Figure 3B,C).

The fLuc and nLuc activity was found to increase over the course of a 7-day bradyzoite conversion period (Figure S3), providing further evidence that luciferase expression is bradyzoite stage-specific. We also found that luciferase expression for both luciferases was measurable in wells seeded with as few as 111 parasites (Figure S3).

In this system, parasite cytosolic fLuc is the variable marker that was predicted to decrease following bradyzoite death, whereas the secreted nLuc is the normalization marker that was predicted to remain static. The readout for bradyzoite viability is therefore ratiometric lum (variable fLuc activity/fixed nLuc activity). We found that ratiometric lum was similar between wells seeded with different parasite numbers, ranging between ~0.0012 and 0.0016 for untreated wells containing 7-days-old bradyzoites (Figure 3C). This is useful in the assay system since it means any variation in the number of parasites seeded per well does not result in a major shift in baseline ratiometric lum, while raw nLuc lum values alone would indicate a parasite load within the monolayer.

Validation of Ratiometric Lum to Establish Parasite Death in *T. gondii* Dual Luciferase-Expressing Bradyzoites. To demonstrate that ratiometric lum decreased in response to lethal compound treatment, both dual luciferase-expressing strains were differentiated to bradyzoites for 7 days in 96-well plates and then subsequently treated for a further 7 days with 20 μM atovaquone (ATQ), 5 μM LHVS, or 0.1% DMSO (vehicle control). Several studies have shown that ATQ has antibradyzoite activity *in vitro* and *in vivo*,[15,25−27] and LHVS was shown to reduce bradyzoite viability *in vitro*.[17] In the BPK1-nLuc strain, ratiometric lum was reduced from a mean of 0.00153 (±0.00023) to a mean of 0.00029 (±0.00016) and 0.00045 (±0.00019) following ATQ and LHVS treatment, respectively (Figure 4A). In the MAG1-nLuc strain, ratiometric lum was reduced from a mean of 0.00136 (±0.00022) to a mean of 0.00029 (±0.00014) and 0.00038 (±0.00012) following ATQ and LHVS treatment, respectively (Figure 4A). Z' factor scores[28] were determined using ratiometric lum values from 20 DMSO-treated sample wells and 20 LHVS-treated sample wells (all within the same plate). Calculated Z' factors were 0.56 and 0.65 for the BPK1-nLuc-myc strain and the MAG1-nLuc-myc strain, respectively (Figure S4).

Bradyzoite qPCR/plaque assays were performed in parallel to the luciferase assays. Both strains formed 26 plaques/1000 genomes on average, which was reduced to less than 1 plaque/1000 genomes following 20 μM ATQ treatment and 4−5 plaques/1000 genomes following 5 μM LHVS treatment (Figure 4B). Therefore, the results of the ratiometric lum assay are comparable to those of a bradyzoite qPCR/plaque assay, whereby both compounds were found to reduce ratiometric lum and the number of plaques/1000 genomes, but ATQ treatment reduced the corresponding values to a greater degree than LHVS in both assays. Since ATQ treatment reduced plaques/1000 genomes to less than 1 in the bradyzoite qPCR/plaque assay, the ratiometric lum values obtained following

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ATQ treatment likely reflect the baseline reading of the assay. Most of all, this validates that a reduction in ratiometric lum correlates with a reduction in bradyzoite viability. Having shown that LHVS is effective in both the DuaLuc and qPCR/plaque assays, we next used it in a time course experiment to determine if, as expected, the differential fLuc activity is responsible for the observed decrease in the fLuc/nLuc ratio during treatment. We found that the fLuc activity increases over time in 0.1% DMSO (vehicle control)-treated samples, whereas fLuc remained diminished in LHVS-treated parasites. Therefore, the distance between fLuc measurements in viable and nonviable parasites became greater over time (Figure 4C). Differently, the nLuc acitivity remains relatively static regardless of treatment (Figure 4C), demonstrating the stability of this signal and utility of nLuc as a normalizer for the cyst load within a well.

Antibradyzoite Compound Testing. A series of compounds were tested for their ability to reduce ratiometric luminescence in the BPK1-nLuc strain, which would indicate their antibradyzoite activity. Of the 11 compounds tested (all at 100 μM), four were found to reduce ratiometric lum with a statistical significance of <0.05 (Figure 5A and Figure S5). These included ATQ (P = 0.0046), LHVS (P = 0.0363), salubrinal (P = 0.0038), and the triazine nitrile TRZ1 (P = 0.0004) (compound 4 in ref 29). Compounds that were found to be ineffective in the assay included chloramphenicol, endochin-like quinolone ELQ316, fluphenazine, hydroxyzine, and sulfadiazine. Intriguingly, guanabenz (100 μM) was found to increase ratiometric luminescence by ∼46% (P = 0.0149) (Figure 5A and Figure S5). Clindamycin treatment was also tested, but this was ineffective at concentrations up to 100 uM, at which point it became toxic to the host cells, resulting in the loss of the monolayer (Figure 5B).

Using Ratiometric Luminescence to Determine Bradyzoite Sensitivity to Compound Treatment. We tested whether the dual luciferase assay could be used to determine bradyzoite sensitivity to a compound by treating parasites with different concentrations of the effective compounds and determining ratiometric luminescence following 7 days of consecutive daily treatment with the different concentrations tested (Figure 5B). This allowed us to determine EC_{50} values within this assay for each of the compounds tested (Table 1). ATQ and LHVS were found to be the most potent antibradyzoite treatments with EC_{50} values of 2.7 and 1.6 μM, respectively, followed by salubrinal (7.9 μM) and TRZ1 (36.7 μM) (Table 1).

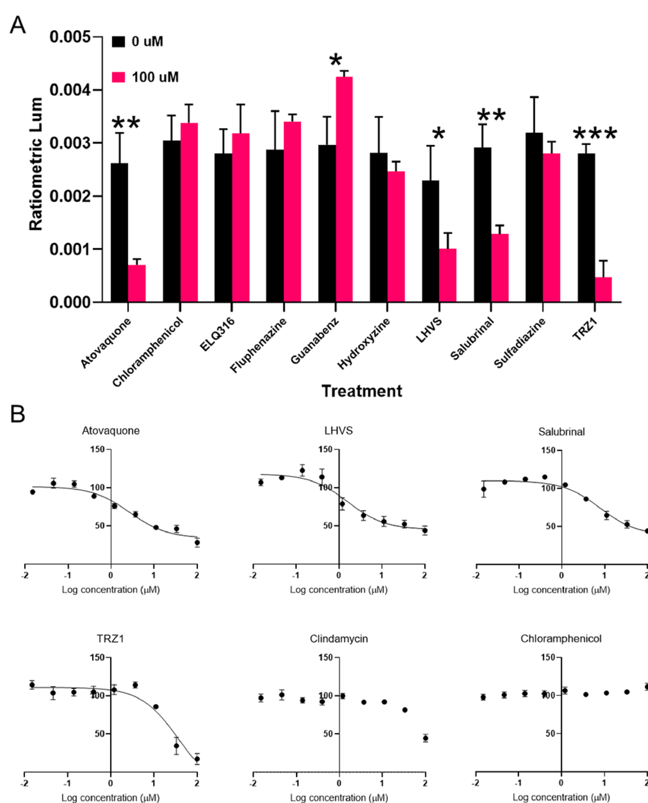

Figure 5. The DuaLuc assay can be used to determine bradyzoite sensitivity to different compounds *in vitro* in a 96-well plate format. (A) Ratiometric luminescence (fLuc/nLuc) following seven days of treatment with 12 different test compounds at 100 μM (or 0 μM test compound, 0.1% DMSO vehicle control). *P < 0.05, **P < 0.005, and ***P < 0.0005 (unpaired t-test). (B) Dose−response curves for each compound found to be effective at reducing ratiometric lum. Chloramphenicol treatment was included as a negative control ineffective at reducing ratiometric lum in *in vitro* bradyzoites. The y-axis represents the ratiometric lum activity relative to vehicle control-treated wells (0.1% DMSO).

Table 1. Summary Table of EC_{50} Values to Determine Relative Sensitivity of Antibradyzoite Compounds Using the Bradyzoite Dual Luciferase Assay

compound	EC_{50} (μM)	95% CI	R^2
atovaquone	2.7	1.4 to 5.5	0.91
LHVS	1.6	0.8 to 3.4	0.85
salubrinal	7.9	4.4 to 14.5	0.91
TRZ1 (triazine nitrile)	36.7	18.3 to 85.7	0.88

■ DISCUSSION

Chronic *T. gondii* infection consists of tissue cysts that contain bradyzoites. Therefore, to test the effectiveness of experimental compounds against chronic infection, a bradyzoite *in vitro* assay is required. The current method for testing bradyzoite viability is the qPCR/plaque assay.[17] This assay involves a 1-to-2-weeks culture period whereby tachyzoites are converted to the bradyzoite life stage. For drug assays, this is then followed by a treatment window (typically 1−2 weeks) before intracellular bradyzoites are harvested from a host cell monolayer. Bradyzoites are extracted from cysts by pepsin digest, and the harvested parasites are placed onto a new monolayer and left undisturbed for up to 14 days to allow plaque formation caused by invaded viable parasites. Therefore, a single experiment can take up to six weeks to perform, which combined with the 6-well plate format renders this approach low-throughput. As a result, we identified a need for a minimum 96-well plate assay to test bradyzoite viability directly in the culture well. Such an assay would need to be sensitive and capable of gaining a signal from a relatively low number of intracellular cysts within a well due to the tendency of *in vitro* cysts to reactivate during the conversion period if infections are too dense. To distinguish between viable and nonviable bradyzoites, the viability marker must also be readily turned over following parasite death. Finally, the markers must be specifically expressed and detectable only in the bradyzoite life stage. To meet these requirements, we developed a dual luciferase-based assay (DuaLuc assay).

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For the DuaLuc assay to be effective at determining bradyzoite viability, it is essential that ratiometric lum decreases as parasites become nonviable. We found this to be the case for both modified strains following treatment with antibradyzoite compounds, including ATQ and LHVS. We also showed that this reduction coincided with a reduction in plaque formation following treatment with the same compounds in a bradyzoite qPCR/plaque assay. A major advantage of the dual luciferase assay in comparison to the qPCR/plaque assay is that it takes approximately half the time to complete, a difference of going from 4−6 weeks per experiment to 2−3 weeks. We noticed that the reduction in ratiometric lum is smaller than the drop observed in qPCR/plaque counts for the same treatments. This is expected since one metric is ratiometric (ratiometric), as opposed to a direct count (plaque count). Since fLuc does not reduce to zero in this assay, there is no "zero" in the ratiometric lum metric, whereas there can be zero plaques in a plaque count. Nevertheless, the reduction in ratiometric lum in nonviable bradyzoites is pronounced and measurable. Using a panel of 11 test compounds, we showed that the dual luciferase assay distinguishes between effective and noneffective compounds. Of those that were effective, we could also determine the sensitivity of parasites to each compound based on relative EC_{50} concentrations. To test these many compounds and concentrations in the 6-well plate qPCR/plaque assays would take months, whereas it is possible to acquire these data within weeks using the dual luciferase assay method, which represents a higher-throughput method. This represents a major improvement on the existing assay and should expedite broader antibradyzoite studies in the future.

Cathepsin L is a major proteolytic cysteine protease in the digestive plant-like vacuole/vacuolar (VAC) organelle in *T. gondii*, and this protease has previously been identified as a potential Achilles' heel for antibradyzoite drug development against the parasite.[17,19,29−31] Consistent with previous reports, the cathepsin L inhibitors tested in the DuaLuc assay here (LHVS and TRZ1) were both effective at reducing ratiometric lum. Both compounds were also recently reported to be effective at reducing bradyzoite viability using the *T. gondii* bradyzoite plaque assay method.[29]

Intriguingly, some compounds were ineffective at reducing ratiometric lum in *in vitro* bradyzoites that have previously been reported to be effective against chronic toxoplasmosis. There were obvious technical issues associated with some compounds, for example, the experimental endochin-like quinolone ELQ316 precipitated in culture wells following application of the treatment. The antibiotic clindamycin is prescribed in combination therapies against the apicomplexans *T. gondii*, *Plasmodium* spp., and *Babesia* spp.[32] However, in the DuaLuc assay, the compound caused lysis of the monolayer at the highest concentration (100 μM) and was found to be ineffective against bradyzoites at lower concentrations. Clindamycin is an inhibitor of protein translation in bacteria. In apicomplexan protozoa, this compound targets the apicoplast ribosome, and treatment with clindamycin ultimately results in the loss of function in this essential organelle.[33] An explanation as to why clindamycin was not found to be effective against bradyzoites in this study can be found in previous observations of clindamycin treatment in *T. gondii* tachyzoites. Clindamycin-treated tachyzoites show a "delayed-death" phenotype, in which parasites in the first lytic cycle (invasion, replication, and egress) remain viable and can mount a subsequent invasion of host cells.[34] It is in the second lytic cycle that parasites then fail to replicate.[34] The bradyzoite luciferase-based assay here is designed to probe parasite viability within the cyst and therefore is unlikely to identify such slow acting compounds for which the effect is only identifiable in subsequent invasion and egress cycles.

Fluphenazine is an antipsychotic that acts on neuronal receptors and has also been reported to have anti-*Toxoplasma* properties.[35] There are contrasting findings on the effects of fluphenazine against *T. gondii*. For example, in HFF cell cultures, fluphenazine treatment has been found to reduce tachyzoite growth at concentrations similar to those used in this study.[35,36] Meanwhile, another study in mice has shown fluphenazine to have no effect on brain cyst burden.[37] This is consistent with our findings here in which fluphenazine was not found to reduce bradyzoite viability in *in vitro* cysts. Therefore, it is possible that the anti-*Toxoplasma* activity of fluphenazine is stage-specific, being effective against tachyzoites but not bradyzoites, or that it is incapable of crossing the cyst wall, which could also be the case for other ineffective compounds.

Guanabenz treatment consistently increased ratiometric luminescence in our assay, indicating an increase in the fLuc viability marker relative to the nLuc normalization marker. This suggests that guanabenz treatment enhanced parasite growth and replication in *in vitro* bradyzoite cysts. This is in contrast to previous *in vivo* studies in mice, in which guanabenz treatment has been found to be effective at reducing (although not eliminating) cyst burden.[38] In a previous study, treatment of *in vitro* bradyzoites with guanabenz alone had little effect on the cyst morphology, although some cysts were reported to contain disorganized parasites.[39] Furthermore, cessation of guanabenz treatment in mice results in a follow-up increase in cyst burden, indicating reactivation in the cysts that survived the treatment period.[38] Guanabenz and salubrinal have both been shown to induce tachyzoite to bradyzoite differentiation and prevent cyst reactivation *in vitro*, by inhibiting TgIF2α dephosphorylation.[40] This suggests a "toxostatic" effect, although to our knowledge, bradyzoite killing following treatment with these compounds is unreported. While guanabenz was found to be ineffective at reducing ratiometric lum in the DuaLuc assay here, salubrinal was effective at reducing the fLuc activity and ratiometric lum compared to vehicle control-treated wells. This might suggest a secondary effect in *T. gondii* bradyzoites by salubrinal that is responsible for reducing parasite viability (*i.e.*, "toxocidal", as opposed to "toxostatic"). It also remains possible that guanabenz stabilizes the ffLuc enzyme, thereby increasing the ratiometric lum value. Direct effects on a reporter are limitations of any enzyme-based assay and necessitate follow-up assays to establish false positives and false negatives.

An active area of *Toxoplasma* research is to identify parasite genes essential to bradyzoite persistence. Although not tested here, it is feasible to consider that the DuaLuc bradyzoite viability assay could be used to identify genes essential for survival in the bradyzoite life stage. The dual luciferase-expressing strains are in a PruΔ*ku80* background, which is defective for nonhomologous recombination, thereby making targeted genetic manipulation more efficient. Therefore, we predict that the dual luciferase-expressing strains would be useful as a parental strain in which to identify on a gene-by-gene basis essential bradyzoite genes by CRISPR/cas9-guided genetic modification.

Although the DuaLuc assay described herein adds a new option for assessing bradyzoite viability, particularly providing a platform for high-throughput screening of drug compounds, the system has some limitations. First, the luciferase assay reagents are quite expensive, which could limit the scope of screening depending on the budget available. Second, the assay is an indirect measure of bradyzoite viability, and thus, it is ideal to use an orthologous approach such as the qPCR/plaque assay to validate findings from DuaLuc screening. Finally, compounds that differentially inhibit fLuc without impairing nLuc will yield false positive results, an outcome that can also be recognized by using an orthologous approach for secondary analysis. Despite these limitations, the DuaLuc system should open new opportunities to screen larger numbers of compounds, thereby improving the odds of identifying those with a potent antibradyzoite activity.

In summary, we report the development of a high-throughput *in vitro* bradyzoite viability assay that can be performed in 96-well plates. We show that the ratiometric luminescence readout of the assay is effective for specifically measuring bradyzoite survival within the cyst. Therefore, this assay facilitates the identification of compounds that impair bradyzoites viability within the cyst. This will be useful in the pursuit of drug therapies against toxoplasmosis aimed at completely eradicating persistent *T. gondii* tissue cysts.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.2c02174.

Additional experimental methods: generation of transgenic *T. gondii* strains and detailed dual luciferase assay protocol; (Table S1) list of primers used in this study; (Table S2) modified cyst-forming *Toxoplasma gondii* strains expressing fLuc and nLuc under bradyzoite-specific promoters; (Figure S1) creation and validation of Pru-*Bag1*/ffLuc-HA-*Ldh2*/MAG1-nLuc-myc and Pru-*Bag1*/ffLuc-HA-*Ldh2*/BPK1-nLuc-myc parasites; (Figure S2) fLuc and nLuc activity in parasites converting to bradyzoites over the course of seven days using the alkaline conversion media method; (Figure S3) luciferase activity across multiple wells of a 96-well plate; (Figure S4) volcano plot of bradyzoite sensitivity to different compounds used in the DuaLuc assay (PDF)

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M.D.C. and V.B.C. conceived the study. All authors designed the research. D.S., M.L., E.B.O., O.H., and M.D.C. performed the research. D.S. analyzed the data. D.S. wrote the paper with contributions from M.D.C. and editing by all authors.

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Notes

The authors declare no competing financial interest.

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