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INHIBITION OF *TOXOPLASMA GONDII* GROWTH BY PYRROLIDINE DITHIOCARBAMATE IS CELL CYCLE SPECIFIC AND LEADS TO POPULATION SYNCHRONIZATION

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Abstract

Successful completion of the *Toxoplasma* cell cycle requires the coordination of a series of complex and ordered processes that results in the formation of two daughters by internal budding. Although we now understand the order and timing of intracellular events associated with the parasite cell cycle, the molecular details of the checkpoints that regulate each step in *T. gondii* division is still uncertain. In other eukaryotic cells, the use of cytostatic inhibitors that are able to arrest replication at natural checkpoints have been exploited to induce synchronization of population growth. Herein, we describe a novel method to synchronize *T. gondii* tachyzoites based on the reversible growth inhibition by the drug, pyrrolidine dithiocarbamate. This method is an improvement over other strategies developed for this parasite as no prior genetic manipulation of the parasite was required. RH tachyzoites blocked by pyrrolidine dithiocarbamate exhibited a near uniform haploid DNA content and single centrosome indicating that this compound arrests parasites in the G1 phase of the tachyzoite cell cycle with a minor block in late cytokinesis. Thus, these studies support the existence of a natural checkpoint that regulates passage through the G1 period of the cell cycle. Populations released from pyrrolidine dithiocarbamate inhibition completed progression through G1 and entered S phase ~2 hours post-drug release. The transit of drug-synchronized populations through S phase and mitosis followed a similar timeframe to previous studies of the tachyzoite cell cycle. Tachyzoites treated with pyrrolidine dithiocarbamate were fully viable and completed two identical division cycles post-drug release demonstrating that this is a robust method for synchronizing population growth in *Toxoplasma*.

Keywords

Apicomplexa; *Toxoplasma gondii*; tachyzoite; cell cycle; pyrrolidine dithiocarbamate

1. Introduction

Toxoplasma gondii is the third leading cause, along with *Salmonella* and *Listeria*, of all deaths due to food-borne disease in the United States [1]. Acquisition of *Toxoplasma* may occur through exposure to contaminated food products or through environmental sources, although recent studies indicate contaminated meat is rare and may be a minor contributor to infection in the U.S. [2]. Inherited differences in the tachyzoite cell cycle that are manifest by distinct cell cycle length [3] influence the severity of clinical disease caused by this pathogen and may

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underlie differences in virulence that are characteristic of the three major genotypic lineages found in Europe and North America [3–5]. Rates of proliferation play a critical role in causing disease pathogenesis in numerous illnesses caused by other members of this phylum including parasites that are responsible for malaria and coccidiosis. Thus, understanding the mechanisms that control parasite division is an important task in the search for new approaches to combat apicomplexan-caused diseases.

T. gondii has evolved cell cycle machinery to produce different modes of replication in the definitive and intermediate hosts (schizogony and endodyogeny, respectively)[6,7], although we do not understand how each cell cycle is regulated or how checkpoints are modified in order to switch between division schemes. Endodyogenic replication of the tachyzoite stage in the intermediate host is a binary process with a single chromosome replication followed by concurrent mitosis and parasite budding to produce new daughters. Chromosome re-replication occurs rarely, but produces viable parasites [8] and might reflect a low frequency switch to multinuclear schizogonous replication, which predominates in definitive life cycle stages. Unlike yeast cell division, tachyzoite budding is fully internal and yields two nearly equal sized daughters. This type of replication has been examined in detail by electron microscopy [9, 10] and using fluorescent markers to allow the visualization of organelle, daughter and nuclear division (reviewed in [7]). Labeling of the major steps of the tachyzoite endodyogeny in terms of conventional eukaryotic organization reveals a cell cycle composed of a primary G1 phase (60%), a bi-modal S (30%) and mitotic/cytokinetic phases (10%) ($G1 > S > M$), while G2 phase is either short or non-existent [3,11,12]. Parasites that possess a late S phase genome content ($\sim 1.8N$) are more frequent than $2N$ parasites [3], which are a small subfraction in asynchronous populations (estimated at 5%; [8]). These results suggest that there is a pause or slowing in late S phase that might represent a novel pre-mitotic checkpoint (equivalent to the G2 checkpoint in animal cells) associated with endodyogeny, although additional proof is needed to verify this model.

Characterization of the *Toxoplasma* cell cycle is greatly aided by the synchronization of population growth. *Toxoplasma*, and other apicomplexa parasites, naturally synchronize the division cycles of progeny that share a vacuole, although vacuolar synchrony begins to break down as parasites reach host cell lysis [8] and this feature alone is insufficient to achieve population synchrony. Metabolic depletion (serum or growth factor starvation) or treatment with growth-inhibitors (e.g. hydroxyurea, aphidicoline, colchicines) are techniques commonly employed to synchronize other eukaryotic cells [13]. Unfortunately, many growth-inhibitors used in animal cells and other protozoa, such as *Plasmodium* [14] and *Leishmania* [15], have not had success in *Toxoplasma*. Chromosome replication is blocked in *Toxoplasma* by the polymerase inhibitors, aphidicoline [16] or hydroxyurea [17], however, these drugs also lead to uncoupling of daughter formation and are lethal. Growth synchrony has been achieved through the use of exogenous thymidine to reversibly block tachyzoites engineered to express the herpes simplex virus thymidine kinase (RH^{TK+}), an enzyme these parasites normally lack. A short treatment of RH^{TK+} tachyzoites with exogenous thymidine, which is known to cause dNTP depletion [18], arrests asynchronous parasite populations in late G1/early S phase and is presumed to act via a checkpoint that governs commitment to chromosome replication in this parasite [3,12].

In this work, we describe a novel method to synchronize *T. gondii* tachyzoite populations that utilizes the antioxidant and metal chelating compound pyrrolidine dithiocarbamate (PDTC). PDTC has previously been used to eliminate extracellular parasites while leaving intracellular parasites unharmed [19]. We provide evidence that PDTC is acting on intracellular parasites to arrest growth primarily in the G1 period of the tachyzoite cell cycle, and demonstrate that a short drug treatment leads to the synchronization of tachyzoites through multiple cell division cycles.

2. Materials and methods

2.1 Cell culture and parasite strains

Human foreskin fibroblasts (HFF) were grown in Dulbecco's modified Eagle medium (DMEM) (Gibco BRL, Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum (Hyclone Laboratories Inc., Logan, UT). All the strains were maintained by serial passage in confluent monolayers of HFF cells according to standard protocols [20]. *Toxoplasma gondii* strain, RH^{TK+}, is a transgenic isolate expressing herpes simplex virus thymidine kinase [12].

2.2 Establishment of synchronous cultures and determination of parasite growth and survivability

To measure parasite replication rates and parasite viability under PDTC treatment (ammonium pyrrolidine dithiocarbamate, Sigma-Aldrich, St. Louis, MO), HFF monolayers were inoculated with tachyzoites at mid-log growth (8–16 parasites per vacuole). Parasite inocula were serially passed twice (24 h growth each passage) to ensure the infected culture was in mid-log growth. Infected HFF monolayers were scraped, passed through a 25 gauge needle, and parasites purified by filtration through 3 µm polycarbonate filters (Costar, Cambridge, MA). Tachyzoites were inoculated into HFF monolayers, incubated for 1 h at 37°C to allow parasite penetration, and then washed with standard growth medium to remove extracellular tachyzoites. Following overnight growth (8–12 h), cultures were treated with PDTC (6 h was the standard PDTC treatment) and then washed 3 times with pre-warmed growth medium (37°C) to release parasites from the growth inhibition. The growth of RH^{TK+} tachyzoites was synchronized with thymidine (10 µM for 4 h) as previously described [12].

Parasite growth was determined for 100 vacuoles in randomly chosen microscopic fields [21]. The average parasite number per vacuole was determined for each time point and doublings calculated by the formula; $\text{Doublings} = \ln [(a.v.s.)_{T_n} \div (a.v.s.)_{T_0}] \div \ln 2$, where $(a.v.s.)_{T_n}$ and $(a.v.s.)_{T_0}$ are the average vacuole size at the time of drug removal (T_0) and following post-drug release (T_n). Parasite survivability was measured by plaque assay; tachyzoites (10^3) were inoculated into HFF monolayers ($T=25\text{cm}^2$), treated with PDTC for 6, 9 or 12 h, washed 3 times, and plaques scored after 8 days of growth at 37°C [20]. The dose of PDTC capable of synchronizing tachyzoite growth must be determined empirically as drug activity was found to vary slightly with each commercial batch or lot synthesis of PDTC (Sigma Co, St Louis, MO.). The optimal dose was defined here as the lowest concentration (60–80 µM for the RH strain) of drug that inhibits parasite growth for at least a 15 h period and yields the highest fraction of daughter formation post-drug release; i.e. >30% of parasites contain internal daughter forms by 4–6h as measured by α IMC1 IFA.

2.3 Flow cytometry and cell cycle analysis

Parasite nuclear DNA content was determined by flow cytometry using propidium iodide (PI) (Sigma, St. Louis, MO) as described previously [12] or using SYTOX Green dye (Molecular Probes #S7020, Invitrogen Corp., Carlsbad, CA)[22], which gives equivalent results to PI staining [7]. Briefly, filter-purified tachyzoites were fixed in 70% (v/v) ethanol with gentle shaking and stored at –20°C for at least 24 h prior to analysis. Fixed parasites were pelleted at $3000 \times g$, resuspended in 50 µM Tris pH 7.5 at a final concentration of 6×10^6 parasites/ml and stained using either PI (0.2 mg/ml final concentration in 0.5 ml total volume) or SYTOX Green (1 µM). RNase cocktail (250U; combination of RNase A, RNase T1, Ambicon Corp., Austin, TX) was added and the parasites incubated in the dark at room temperature for 30 min. Nuclear DNA content was measured based on PI (FL-2) or SYTOX Green (FL-1) fluorescence using a 488 nm argon laser (Becton-Dickinson Inc., San Jose, CA). Fluorescence was collected in linear mode (10,000 events) and the results were quantified using CELLQuestTM v3.0

(Becton-Dickinson Inc.). The percentages of G1 (1N), S (1-2N) and G2 + M (2N) tachyzoites were calculated based on defined gates for each population.

2.4 Immunofluorescence assays (IFA) for cell cycle markers

Fluorescent patterns of inner membrane complex (IMC1, to assess daughter formation)[23], proliferating cellular nuclear antigen (PCNA1, to assess nuclear morphology)[3], centrin (to assess centrosome duplication)[24], and genomic DNA (stained with dapi) were evaluated by IFA in tachyzoites grown in HFF monolayers (9cm² slideflask, #170920, Nalge Nunc International, Roskilde, Denmark) and synchronized by PDTC treatment. Microscopic slideflasks were used throughout these studies to better match the culture format employed in parallel experiments to determine DNA content by FACS analysis. HFF monolayers were inoculated with 7×10⁵ mid-log tachyzoites and the parasites allowed to invade for 1 h. The slideflasks were washed using standard DMEM growth media to remove extracellular tachyzoites, and then incubated at 37°C for 10 h prior to PDTC treatment (average vacuole size of two parasites for RH strain). PDTC block for 6 h and then release by washing in warm media followed the same protocol as used for DNA analysis. At various intervals, the infected slideflasks were washed three times in 1x PBS, fixed with 3.7% paraformaldehyde in 1x PBS and permeabilized with in 0.25% Triton X-100 in 1x PBS for 10 min. Parasites were incubated for 1 h with one or a combination of primary antibodies: monoclonal mouse anti-IMC1 antibody (1:2000, a gift from Dr. Ward, University of Vermont), monoclonal mouse anti-centrin antibody 26-14.1 for centrosome (1:2000, a gift from Dr. Salisbury, Mayo Clinic, Rochester)[24] and a polyclonal rabbit anti-PCNA1 antiserum (1:5000)[25]. Slideflasks were washed three times in 1x PBS and stained with secondary antibodies for 1 h in the dark; goat-anti-rabbit and a goat-anti-mouse antibodies conjugated with Alexa Fluor 488 (FITC filters) or Alexa Fluor 594 (TRITC filters) dyes (Molecular Probes, Eugene, OR, USA). Genomic DNA was visualized by 0.1 mg/ml DAPI staining (2-(4-amidinophenyl)-6-indolecarbamidinedihydrochloride #D8417, Sigma-Aldrich, St. Louis, MO) added during incubation with secondary antibody. The slideflasks were washed four times in 1x PBS, the gaskets from the flasks were removed, and the slides mounted with Gel-mount solution containing 2.5% (w/v) diazabicyclo[2.2.2.7.]octane. Parasites were evaluated with an epifluorescence microscope (Eclipse TE300, Nikon Inc., Melville NY) and images were collected with a digital camera (SPOTTM, Dynamic Instruments Inc., Sterling Heights MI).

3. Results

3.1 PDTC inhibition of tachyzoites is cell cycle specific

The cytostatic property of growth inhibitors is often associated with arrest at a natural checkpoint in yeast and animal cells [26] and this feature has been exploited to synchronize eukaryotic cell populations. Hydroxyurea and aphidicolin are two examples of this type of compound, although these, and other commonly used inhibitors, have proven to be cytotoxic to *Toxoplasma* parasites [16,17]. It is therefore important to identify drugs whose effects on parasite growth are reversible and determine whether growth inhibition is cell cycle specific. The antioxidant and metal chelating compound PDTC has been shown to inhibit cell cycle progression (G1-specific) and DNA synthesis of vascular smooth muscle cells through a mechanism that involves the down regulation of cyclin/CDK activities and increased expression of the CDK inhibitor p21 [27]. In *Toxoplasma* tachyzoites, this compound is toxic only to extracellular parasites, whereas the effect on intracellular parasite growth is cytostatic [19]. To investigate the cell cycle context of PDTC inhibition in *Toxoplasma*, we have examined in detail the characteristics of parasite growth at various drug concentrations (Table 1). Following an overnight period of growth (10 h overnight growth vacuoles contained predominantly two parasites, Table 1), tachyzoites were exposed to PDTC for relatively short periods (6 h was used for all block & release experiments and is based on the estimated 6–7 h

doubling for RH strains used in these studies)[3]. Changes in vacuole size were followed in drug-treated cultures, and in cultures where the drug was removed, and drug toxicity was also reevaluated by plaque assay (only results of 80 μ M PDTC are shown). The results in Table 1 show that PDTC inhibition of parasite growth was dose dependent and characterized by high survivability (>94%) of parasites that were growth inhibited for a duration of up to 12 h (plaque assay results bottom of Table 1). These data are consistent with the previous report demonstrating that intracellular PDTC treatment has a cytostatic effect on tachyzoite growth [19].

Monitoring the change in vacuole size can provide important information about whether PDTC treatment inhibits parasite growth generally or at a specific place in the cell cycle. If PDTC results in a specific cell cycle block, we would expect a rapid growth arrest within the same (if parasites are behind the block) or the next cell cycle (if parasites are past the block), and upon drug release, the population growth should follow stepwise kinetics. RH tachyzoites exposed to PDTC at or below 40 μ M did not appear to be fully growth arrested (note the slow increase in the numbers of parasites per vacuole during 15 h block at 40 μ M versus 80 μ M PDTC) and when drug was removed from cultures treated with 40 and 20 μ M PDTC the change in the number of parasites per vacuole followed a more asynchronous pattern than a stepwise increase (e.g. compare 20 μ M PDTC to asynchronous growth, Table 1). By contrast, changes in the growth of infected cultures treated with 80 μ M PDTC showed a stepwise growth kinetics that would be consistent with a cell cycle specific block. Partial cell cycle progression occurred under the 80 μ M PDTC block with ~75% of 2-vacuoles (58 of 80 total, Table 1) progressing to 4-vacuoles during the 6 h block, but vacuole size was held constant thereafter (up to 15 h). This suggests that at the time of drug addition 20–30% of the population was behind the PDTC block and did not progress to the next cell cycle. When parasites were released from 80 μ M PDTC, the population divided stepwise with 50 of 80 vacuoles containing four parasites dividing to become vacuoles of 8 parasites between 3 h and 7 h post-drug release (doubling of the average vacuole size from 3.5 to 6.5 over a period equal to the estimated cell cycle length of 7 h also indicates the population is dividing synchronously). At the conclusion of these experiments the vacuole size distribution of populations treated with 80 μ M PDTC were much tighter (two vacuole sizes) than either asynchronous controls or populations treated with 20 μ M PDTC (Table 1). At higher PDTC concentrations (including 0.1 and 1 mM PDTC, not shown), the pattern of parasite growth also appeared consistent with a cell cycle specific block, but the timeframe of release was extended indicating that higher drug concentrations slowed the overall growth rate of the released populations. The optimal PDTC dose required to induce growth synchrony was found to vary slightly (60–80 μ M), but was consistent within a commercial batch of drug, and thus, the optimal dose of PDTC was empirically determined for each drug lot (data not shown).

3.2 The primary PDTC block occurs in the G1 phase of the tachyzoite cell cycle

The mechanisms of checkpoint regulation of apicomplexan cell cycle are largely not understood. However, previous experimental studies in *T. gondii* have demonstrated that tachyzoites can specifically arrest in G1, at the G1/S boundary, or in mitosis [12,28,29] indicating the *Toxoplasma* cell cycle is coordinated at multiple places. In order to determine where in the cell cycle PDTC arrests parasite growth, we compared the DNA content of asynchronous untreated populations with parasites whose growth was blocked by PDTC (Fig. 1B and C). Previous studies established that randomly growing populations have a bimodal distribution with peak DNA contents of haploid (~1N) and near-diploid (~1.8N), although parasites containing a DNA content falling between 1-1.8N and 1.8-2N are also present in these populations [3,12]. Consistent with these earlier studies, we found the full range of DNA contents (from 1-2N) in tachyzoite populations obtained from asynchronous cultures (Asyn populations in histogram panels in Fig. 1A and B and also blue dot plot in Fig. 1C). By contrast,

populations obtained from 6 h PDTC treated cultures (Fig. 1B histogram and 1C, red dot plot) were primarily 1N (85%) with a small fraction (15%) containing a 1.8-2N DNA content that was comprised of late cytokinetic stages with duplicated centrosomes (not shown). Notably reduced in PDTC treated populations were S phase parasites, whose DNA contents are intermediate between 1N and 1.8N (note the lack of red dots in the oval region within the overlay of the asynchronous-PDTC plots corresponding to early-mid S phase, Fig. 1C). To confirm the haploid growth arrest by PDTC, we determined that 85% of parasites in this blocked population contained a single centrosome. By contrast to the PDTC-treatment, thymidine-blocked transgenic RH^{TK+} parasites are stopped at the late-G1/early S phase boundary and the majority of these parasites (67%) have a duplicated centrosome [29]. Combined, these results indicate that PDTC-treatment primarily stops parasite growth primarily in the G1 phase of the cell cycle, which is remarkably similar to the reported effects of PDTC on animal cells [27], and distinct from the thymidine-block of RH^{TK+} parasites.

3.3 PDTC versus thymidine synchronization of the tachyzoite cell cycle

In order to better understand the nature of the G1 arrest caused by PDTC, we compared the cell cycle progression of RH tachyzoites synchronized with PDTC media to the previously published thymidine-synchrony model [12]. Asynchronous populations of RH and RH^{TK+} strains were very similar with respect to average doubling time, genomic DNA profiles, and the length of mitosis as estimated by the subpopulation containing intracellular budding forms (10–12% of parasites growing asynchronously have internal daughter forms as assessed using anti-IMC1 antibodies, not shown)[3].

As reported previously [12], RH^{TK+} parasites arrested by a short treatment (4 h) with 10 μ M thymidine and then released into fresh media, advance through the cell cycle within a ~2 h window of synchrony. Because thymidine arrests RH^{TK+} tachyzoites at G1-S phase boundary [3,12] (67% have already duplicated their centrosomes), parasites released from the thymidine block immediately progressed through S phase (Fig. 2A, R1-R3 panels) and completed mitosis and cytokinesis within a timeframe that is consistent with the combined duration of S phase and mitosis/cytokinesis in these parasites (Fig. 2A, R0-R4)[3,12].

We found that parasites synchronized with PDTC must complete G1 before they enter S phase at 2–3 h post drug-release (Fig. 2B R2-3, R3 shown only). Because there is no genomic DNA change during G1, we observed little or no alteration in the DNA profiles of PDTC-synchronized populations during the first 2 h of release from the drug. Once PDTC-synchronized populations cross the G1/S boundary (~R2-3 post-release), the genomic DNA content changes (Fig. 2B, R3-R7) are identical to synchronized RH^{TK+} parasites indicating the length of S phase and mitosis and the window of synchrony are similar in these models (Fig. 2A and B; compare the DNA content changes of the thymidine model from R1-R5 to the PDTC model from R3-R7). These results further validate that PDTC inhibited parasites growth at a point in G1 that is further from S phase entry than the late G1/S thymidine growth arrest of RH^{TK+} parasites.

3.4 PDTC-treated populations remained synchronized through two division cycles

Because of the relative tight synchrony induced by PDTC-block (estimated to be synchrony in a ≤ 2 h cell cycle window as compared to a 7 h cell cycle length), we explored whether populations remained synchronous through multiple cell cycles. RH parasites that were blocked with PDTC and then released into fresh media were monitored for changes in DNA content over a period of 16 h (Fig. 3). To increase the confidence of these results, we employed a newer DNA fluorescent stain that has been reported to provide more reliable DNA content changes in yeast cells [22]. When used with *Toxoplasma* tachyzoites, SYTOX Green has been shown to give equivalent DNA profiles to the standard PI method, although there is

substantially higher fluorescent signal from SYTOX Green stained samples [7]. Using this new staining method, we found that PDTC-synchronized populations had a unimodal DNA content (1N) at 8 h and 16 h post-PDTC release consistent with completion of two cell cycles during the course of this experiment. The transit time for each cell cycle was identical, and thus, at intermediate time points the DNA profiles of 4 h post-released parasites (R4) reasonably matched 12 h post-release (R12), while R6 matched R14 and R8 matched R16. Because the window of synchrony is ~2 hours and S phase and mitosis are relatively short [3,12], the G1 peak reduces dramatically but does not fully disappear before new parasites emerge as indicated by the increase in 1N parasites at R6 and R14. We also noted that the rightward shift to higher DNA fluorescence in the second DNA peak at R6 and R14 indicated that the population is moving synchronously through S and then into mitosis (Fig. 3, compare the second fluorescent peak at R6 and R14 profiles to R4 and R10). A major stepwise increase in parasite numbers in these cultures was coincident with transition into each cell cycle and the population doubling twice during the course of this experiment (PDTC-synchronized average vacuole size at R0=2.8 parasite per vacuole; R16=10.0 parasites per vacuole).

3.5 PDTC-induced synchronization affects parasite size and increases the discard of cytoplasm into the parasitophorous vacuole

During microscopic examination of parasites blocked and released from PDTC containing medium, we noticed an apparent affect on cell volume as parasites appeared wider and rounder than asynchronous controls (this was also seen in haploid parasites in the FACS overlay of these populations, Fig. 1C). This observation was confirmed by the larger and more variable forward scatter (FSC) detected in FACS analysis of populations released from PDTC-block as compared to RH^{TK+}-populations synchronized by a 4 h thymidine-block (Fig. 4 legend see FCS values for 1N gated parasites). The FSC of 1N parasites formed at 6 h (R6) in PDTC-synchronized populations were smaller than parasites containing a haploid DNA content at 4 h post-PDTC release indicating that there was a substantial decrease in cell volume associated with the formation of new daughter parasites (R6, Fig 4A). Newly formed parasites (early G1) in thymidine-synchronized populations were also smaller, however, when compared to 1N parasites in late G1 that were present at earlier time points these changes were less dramatic (compare FSC values for 1N parasites at R3 versus R5 in Fig. 4 legend).

Representative microscopic examples of parasite budding in asynchronous controls and in cultures following release from a PDTC block are shown in Fig. 4B. The decrease in cell size observed by FACS (Fig. 4A, top panel R6) was coincident with daughters budding out of the mother cell rather than fully consuming the mother. There was substantial residual mother cytoplasmic mass in the vacuoles of PDTC-synchronized populations that were forming new daughters when compared to controls (see arrows in Fig. 4B). This material was discarded into the parasitophorous vacuole and stained positively with antibodies against IMC1 but did not stain with dapi indicating that nuclear material had been distributed into each daughter parasite. Cytoplasmic discard also occurred in the controls (Fig. 4C, black bars), although this was relatively infrequent (<3%). There was a significant increase in vacuoles containing IMC1-positive discard (Fig. 4C, black bars) that kinetically followed the rise in internal daughter forms (Fig. 4C, daughters peak at R4 in this time course, grey bars) and reached a peak as parasites completed the first cell cycle following drug-release (Fig. 4C, peak discard R8, black bars; stepwise division R4-R8, line graph). Evidence of cytoplasmic-discard is greatly reduced at the conclusion of the next cell cycle indicating that the discarded material is resorbed by the vacuole at the end of the next cycle and this was consistent with the restoration of a normal parasite size in 16 h post-release populations (data not shown).

4. Discussion

In this study, we have introduced a novel protocol for establishing synchronous *Toxoplasma* populations that is based on the reversible growth inhibition of the drug PDTC. PDTC protocols offer advantages over an earlier synchrony model that is dependent on ectopic expression of thymidine kinase [12] as PDTC is capable of inhibiting the growth of all major genotypic strains (Type I–III, data not shown) without genetic manipulation. This model will mediate efforts to further investigate the basic features of endodyogenic replication, will help define the differences in cell cycle progression that vary in distinct strains, and will aid in the discovery of parasite gene expression that is under cell cycle control during asexual replication.

The cell cycle progression of tachyzoite populations exposed to a limited PDTC-block and then released into fresh media appears to be similar to thymidine-induced synchrony [3,12] with the position of growth arrest and the absolute timing of S phase entry distinctive for each model. Thymidine-arrest of RH^{TK+} -parasites occurs at the G1/S boundary and is consistent with the effect of high thymidine concentrations in animal cells, which causes dNTP depletion and growth arrest at a similar position in the animal cell cycle [18]. Thymidine blocked parasites possess a broad 1N to 1.2N DNA content, duplicated centrosomes, and they immediately progress through S phase when released into fresh media [3,12]. By contrast, PDTC arrests tachyzoites with a near uniform haploid DNA content and single centrosome with a small fraction of parasites containing a 1.8-2N DNA content that is proportionally equivalent to parasites with late internal daughters in these arrested populations (not shown). Whereas thymidine-arrested populations immediately progress through S phase, entry into S phase is delayed for PDTC-blocked parasites that are released from drug, although once committed to chromosome replication, PDTC-treated parasites complete S phase and mitosis in a timeframe that is equivalent to parasites synchronized with thymidine. These results are consistent with PDTC arresting parasites in early G1 with a minor block in late cytokinesis (parasites had not yet separated from the mother cell). PDTC is known to inhibit the growth of smooth muscle cells through a mechanism involving the down regulation of G1 cyclin/CDK complexes and the increased expression of the cyclin kinase inhibitor, P21 [27]. While the target of PDTC inhibition in *Toxoplasma* is unknown, the similarities in cell cycle inhibition suggest PDTC might be acting in *Toxoplasma* through the inhibition of a similar G1 checkpoint mechanism. It is possible that PDTC acts indirectly through a primary host cell target, however, pre-treatment of HFF cells with PDTC for varying time periods caused no growth inhibition of parasites that subsequently invaded the pre-conditioned monolayer (data not shown).

Reversible growth arrest of tachyzoites in G1 supports a new checkpoint in *Toxoplasma* that may be related to chemical mutants that show reversible, conditional arrest with a haploid DNA content (Jerome, Conde, and White, unpublished results) [28] and these results join other experimental evidence for cell cycle controls in *Toxoplasma* including the late-G1/early S phase checkpoint in thymidine-arrest (see above and [12]) and mechanisms that coordinate mitosis and cytokinesis. Linkage between nuclear division and daughter formation is observed in the mitotic mutant, ts11C9 [7,29], which is genetically complemented by a known suppressor of cyclin kinase activity [29], and in tachyzoites that undergo rare chromosome re-replication where daughter formation is paused so that viable parasites are produced at the conclusion of the second round of DNA replication [8]. Altogether, these results give a partially answer to the question of whether checkpoints are active in *Toxoplasma*, but the total number of checkpoints and the details of each mechanism is still uncertain. The *Toxoplasma* genome contains up to six putative cyclin genes for which RNA expression is detectable for five of these cyclins in the tachyzoite based on microarray analysis using the new Affymetrix *Toxoplasma* GeneChip (Kvaal, Conde, and White, unpublished results). Furthermore, checkpoint mechanisms in this parasite are likely conserved as there are similar or greater numbers of cyclin homologs in the genomes of other apicomplexa parasites (reviewed in [7]).

Cell cycle regulation in yeast limits the size distributions of growing yeast populations due to the tight coupling between cell growth and cell division present in this organism [30]. Previous studies have shown that yeast divide and correct the large cell size by decreasing the length of the next G1 phase or by lengthening the next G1 for the smaller daughter cell [31]. Tachyzoites held under PDTC block show increased cell size as they approached the first division following drug release, however, unlike yeast mechanisms that would predict a shorter cell cycle as cell volume increases, tachyzoites released from PDTC block replicate with a cell cycle length that is similar to asynchronous populations and this cell cycle timing is precisely maintained through at least two cell divisions in PDTC synchronized populations. Thus, our data suggest that these parasites do not control cell volume by altering cell cycle progression, but rather through a mechanism of cytoplasmic discard, which was first described in 1958 [32], and is routinely observable at low levels in tachyzoite cultures. Our observations also indicate that complete consumption of the mother cell may not be required for parasite budding and raises questions as to whether the outer membrane is in fact made primarily de novo. This seemingly wasteful mechanism likely arose as the result of the adaptation of this protozoan to a parasitic life style where conservation of energy and macromolecular synthesis is less vital to survival and is probably not coupled to other cell cycle checkpoints.

In summary, more research is needed to resolve the cell cycle transitions that govern critical steps from late S phase through mitosis in *Toxoplasma* and to understand how the complement of cyclin-CRK complexes act to regulate these transitions. These studies will be aided by the establishment of new models such as the PDTC-induction of synchrony reported here, but additional informative mutants are also needed to determine the existence of checkpoints and identify the factors regulating S phase progression, chromatin condensation, spindle assembly and chromosome segregation in anaphase. Given the critical importance of parasite burden to disease pathogenesis, understanding checkpoint mechanisms in these parasites may offer novel targets upon which to develop new therapeutic interventions.

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Abbreviations

PDTC	pyrrolidine dithiocarbamate
PCNA	proliferating cell nuclear antigen
IMC1	inner membrane complex 1
HFF	human foreskin fibroblasts

References

1. Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, Shapiro C, Griffin PM, Tauxe RV. Food-related illness and death in the United States. *Emerg Infect Dis* 1999;5:607–625. [PubMed: 10511517]
2. Dubey JP, Hill DE, Jones JL, Hightower AW, Kirkland E, Roberts JM, Marcet PL, Lehmann T, Vianna MC, Miska K, Sreekumar C, Kwok OC, Shen SK, Gamble HR. Prevalence of viable *Toxoplasma*

- gondii* in beef, chicken, and pork from retail meat stores in the United States: risk assessment to consumers. *J Parasitol* 2005;91:1082–1093. [PubMed: 16419752]
3. Radke JR, Striepen B, Guerini MN, Jerome ME, Roos DS, White MW. Defining the cell cycle for the tachyzoite stage of *Toxoplasma gondii*. *Mol Biochem Parasitol* 2001;115:165–175. [PubMed: 11420103]
 4. Saeij JP, Boyle JP, Collier S, Taylor S, Sibley LD, Brooke-Powell ET, Ajioka JW, Boothroyd JC. Polymorphic secreted kinases are key virulence factors in toxoplasmosis. *Science* 2006;314:1780–1783. [PubMed: 17170306]
 5. Taylor S, Barragan A, Su C, Fux B, Fentress SJ, Tang K, Beatty WL, Hajj HE, Jerome M, Behnke MS, White M, Wootton JC, Sibley LD. A secreted serine-threonine kinase determines virulence in the eukaryotic pathogen *Toxoplasma gondii*. *Science* 2006;314:1776–1780. [PubMed: 17170305]
 6. Speer CA, Dubey JP. Ultrastructural differentiation of *Toxoplasma gondii* schizonts (types B to E) and gamonts in the intestines of cats fed bradyzoites. *Int J Parasitol* 2005;35:193–206. [PubMed: 15710440]
 7. White, MW.; Radke, JR.; de Felipe, MC.; Lehmann, M. Chapter 15: Cell cycle control/parasite division. In: Aijoka, J.; Soldati, D., editors. *Toxoplasma: Molecular and Cellular Biology*. Norwich, U.K: Horizon Scientific Press; 2007. p. 263-282.
 8. Hu K, Roos DS, Angel SO, Murray JM. Variability and heritability of cell division pathways in *Toxoplasma gondii*. *J Cell Sci* 2004;117:5697–5705. [PubMed: 15494366]
 9. Ogino N, Yoneda C. The fine structure and mode of division of *Toxoplasma gondii*. *Arch Ophthalmol* 1966;75:218–227. [PubMed: 4159376]
 10. Sheffield HG, Melton ML. The fine structure and reproduction of *Toxoplasma gondii*. *J Parasitol* 1968;54:209–226. [PubMed: 5647101]
 11. Cornelissen AW, Overdulve JP, van der Ploeg M. Determination of nuclear DNA of five eucoccidian parasites, *Isospora (Toxoplasma) gondii*, *Sarcocystis cruzi*, *Eimeria tenella*, *E. acervulina* and *Plasmodium berghei*, with special reference to gamontogenesis and meiosis in *I. (T.) gondii*. *Parasitology* 1984;88(Pt 3):531–553. [PubMed: 6204268]
 12. Radke JR, White MW. A cell cycle model for the tachyzoite of *Toxoplasma gondii* using the *Herpes simplex* virus thymidine kinase. *Mol Biochem Parasitol* 1998;94:237–247. [PubMed: 9747974]
 13. Hung DT, Jamison TF, Schreiber SL. Understanding and controlling the cell cycle with natural products. *Chem Biol* 1996;3:623–639. [PubMed: 8807895]
 14. Inselburg J, Banyal HS. *Plasmodium falciparum*: synchronization of asexual development with aphidicolin, a DNA synthesis inhibitor. *Exp Parasitol* 1984;57:48–54. [PubMed: 6420180]
 15. Soto M, Iborra S, Quijada L, Folgueira C, Alonso C, Requena JM. Cell-cycle-dependent translation of histone mRNAs is the key control point for regulation of histone biosynthesis in *Leishmania infantum*. *Biochem J* 2004;379:617–625. [PubMed: 14766017]
 16. Shaw MK, Roos DS, Tilney LG. DNA replication and daughter cell budding are not tightly linked in the protozoan parasite *Toxoplasma gondii*. *Microbes Infect* 2001;3:351–362. [PubMed: 11369271]
 17. de Melo EJ, Mayerhoffer RO, de Souza W. Hydroxyurea inhibits intracellular *Toxoplasma gondii* multiplication. *FEMS Microbiol Lett* 2000;185:79–82. [PubMed: 10731610]
 18. Terasima T, Tolmach LJ. Growth and nucleic acid synthesis in synchronously dividing populations of HeLa cells. *Exp Cell Res* 1963;30:344–362. [PubMed: 13980637]
 19. Camps M, Boothroyd JC. *Toxoplasma gondii*: selective killing of extracellular parasites by oxidation using pyrrolidine dithiocarbamate. *Exp Parasitol* 2001;98:206–214. [PubMed: 11560413]
 20. Roos DS, Donald RG, Morrisette NS, Moulton AL. Molecular tools for genetic dissection of the protozoan parasite *Toxoplasma gondii*. *Methods Cell Biol* 1994;45:27–63. [PubMed: 7707991]
 21. Jerome ME, Radke JR, Bohne W, Roos DS, White MW. *Toxoplasma gondii* bradyzoites form spontaneously during sporozoite-initiated development. *Infect Immun* 1998;66:4838–4844. [PubMed: 9746587]
 22. Haase SB, Reed SI. Improved flow cytometric analysis of the budding yeast cell cycle. *Cell Cycle* 2002;1:132–136. [PubMed: 12429922]
 23. Hu K, Mann T, Striepen B, Beckers CJ, Roos DS, Murray JM. Daughter cell assembly in the protozoan parasite *Toxoplasma gondii*. *Mol Biol Cell* 2002;13:593–606. [PubMed: 11854415]

24. Striepen B, Crawford MJ, Shaw MK, Tilney LG, Seeber F, Roos DS. The plastid of *Toxoplasma gondii* is divided by association with the centrosomes. *J Cell Biol* 2000;151:1423–1434. [PubMed: 11134072]
25. Guerini MN, Behnke MS, White MW. Biochemical and genetic analysis of the distinct proliferating cell nuclear antigens of *Toxoplasma gondii*. *Mol Biochem Parasitol* 2005;142:56–65. [PubMed: 15878790]
26. Murray, JM.; Hunter, CA. *The Cell Cycle an introduction*. Oxford University Press; 1993.
27. Moon SK, Jung SY, Choi YH, Lee YC, Patterson C, Kim CH. PDTC, metal chelating compound, induces G1 phase cell cycle arrest in vascular smooth muscle cells through inducing p21Cip1 expression: involvement of p38 mitogen activated protein kinase. *J Cell Physiol* 2004;198:310–323. [PubMed: 14603533]
28. Radke JR, Guerini MN, White MW. *Toxoplasma gondii*: characterization of temperature-sensitive tachyzoite cell cycle mutants. *Exp Parasitol* 2000;96:168–177. [PubMed: 11162367]
29. White MW, Jerome ME, Vaishnav S, Guerini M, Behnke M, Striepen B. Genetic rescue of a *Toxoplasma gondii* conditional cell cycle mutant. *Mol Microbiol* 2005;55:1060–1071. [PubMed: 15686554]
30. Hatzis C, Porro D. Morphologically-structured models of growing budding yeast populations. *J Biotechnol* 2006;124:420–438. [PubMed: 16516320]
31. Hartwell LH, Unger MW. Unequal division in *Saccharomyces cerevisiae* and its implications for the control of cell division. *J Cell Biol* 1977;75:422–435. [PubMed: 400873]
32. Goldman M, Carver RK, Sulzer AJ. Reproduction of *Toxoplasma gondii* by internal budding. *J Parasitol* 1958;44:161–171. [PubMed: 13539708]

Figure 1. PDTC and thymidine treatments block parasite growth at different points in the cell cycle (A). RH^{TK+} parasites grown overnight at 37°C were treated with media containing 10 μ M thymidine for 4 h. Samples were then processed for DNA analysis using established protocols (70% EtOH fixation) and parasites were stained with propidium iodide. DNA content was determined for drug-treated and asynchronous (Asyn) controls by flow cytometry in FL-2 and 10,000 events were collected for each histogram. Fluorescence results (excitation 564–606 nm) collected in linear mode (400 mode calibration for 1N) was quantified using CELLQuestTM v3.0 software and the percentages of parasites containing haploid (1N), intermediate, and diploid (2N) genome equivalents were calculated using defined gates. Note that thymidine-inhibition results in a broad asymmetric peak corresponding to a 1N to 1N+ DNA content. In parallel cultures, RH^{TK+} parasites in 100 vacuoles were evaluated for the fraction of single versus duplicated centrosomes in the blocked population by IFA with 60% single/40% double centrosomes in the asynchronous population versus 33% single/67% double centrosomes in the thymidine-blocked population.

(B & C). RH tachyzoites, were grown at 37°C for 10 h (average vacuole size of 2) and then normal growth medium or medium containing 80 μ M PDTC was added and the cultures incubated for 6 h at 37°C. Samples were then harvested for DNA content analysis of asynchronous controls versus PDTC-blocked populations as described above. Genomic distributions collected in FL-2 for asynchronous (Asyn) and PDTC-blocked parasites were graphed in a histogram plot (B), while an overlay plot (C) of forward scatter (FSC-H) versus FL-2 fluorescence intensity (1N=400 and 2N=800 mode fluorescence) was generated for asynchronous controls (blue dot plot) and the 6 h PDTC inhibited samples (red dot plot). A substantial fraction of parasites (85%) blocked with PDTC had a haploid DNA content (symmetrical distribution) by FACS (Fig. 1B) and in parallel PDTC-blocked cultures there was an equal fraction of parasites that contained a single, unduplicated centrosome (85%). A small fraction of the PDTC blocked population contained duplicated centrosomes (15%), which corresponds to the fraction of parasites with 1.8N-2N DNA contents, and also to the frequency of mature daughter forms in this population (not shown) indicating that PDTC has a secondary minor block in late cytokinesis. Parasites with DNA contents intermediate between

1N and 1.8 N were significantly diminished in the PDTC-treated cultures indicating there were few S phase parasites in the PDTC-blocked samples (in the oval region containing early to mid S phase parasites, there are very few red dots). Note also that PDTC-blocked parasites with a haploid DNA content had a larger forward scatter as compared to asynchronous controls.

Figure 2. Thymidine- and PDTC-synchronized populations progress through S phase and mitosis in a similar timeframe

(FACS panel A). RH^{TK+} parasite cultures were blocked with 10 μ M thymidine for 4 h as described in Fig. 1. After the drug treatment, the parasites were washed 3x in warm medium in order to release them from the drug treatment. At various times post drug-release, samples were harvested for the analysis of DNA content by FACS using the PI stain. As described previously [12], thymidine-arrested RH^{TK+} parasites are blocked at the G1/S phase boundary (R0) and immediately enter S phase upon drug-release (R1). New daughter parasites start emerging by 3 h post-drug release (R3), and the entire population begins to enter the S phase of the next cell cycle by 7 h post-release (not shown)[12].

(FACS panel B). PDTC-block of RH tachyzoites was established as described in Fig. 1 and the population released by washing 3x with warm medium. FACS analysis of the DNA content (PI stain) of populations released from drug was performed on samples taken every hour through seven hours post-drug release. There was little change in the DNA distributions of populations through 2 h post-drug release (R1 shown only) suggesting the parasites were progressing through the G1 phase. Between 2–3 h post-release parasites containing a 1N+ content were apparent (R3 shown only) indicating that parasites released from PDTC inhibition enter S phase 2–3 h post-drug release. The progressive changes in DNA content from R3-R7 that lead to new daughter parasites (R3 in panel A and R5 in panel B) were very similar to RH^{TK+} populations synchronized with thymidine treatment indicating that chromosome replication and segregation followed an identical timeframe in each method of synchronization.

Figure 3. A short PDTC block synchronizes population growth through at least two division cycles Mid-log (4–8 parasites per vacuole) RH tachyzoites were inoculated into HFF cells, grown overnight, blocked for 6 h with PDTC, and then released by washing the cultures in drug-free medium. Parasites were harvested every two hours for 16 h following release from drug (R2–R16), and DNA content determined using the SYTOX Green dye. Fluorescent results from 10,000 events collected in FL1 were plotted in a series of individual histograms aligned here with respect to the 1N DNA content in each graph. The DNA content distribution of asynchronous growing population (Asyn) was harvested from a control culture at R16. Arrows indicate 1 N (peak mode FL1 = 400) and 2 N (FL1 = 800) DNA contents. Changes in the DNA content of parasites in the first division cycle post-drug release paralleled earlier experiments

with new daughter parasites emerging between 4–6 h release (R4 and R6) and the population fully in G1 by 8 h post-drug release (R8). This pattern of changes in genomic DNA content is then repeated in the next 8 h (R8-R16) with the DNA profile of R12 equal to R4, R14 matched to R6, and R16 similar to R8. Note that between the R4/R12 and R6/R14 time points the second fluorescent peak (second arrow) shifts such that in the R6 and R14 populations there is a true diploid subpopulation indicating that the synchronized population has now fully passed through S phase and the lagging edge of the population is in mitosis/cytokinesis.

Figure 4. PDTC-inhibition leads to increased parasite size and cytoplasmic discard into the parasitophorous vacuole

(A). Genomic DNA profile versus FSC of PDTC-synchronized RH parasites and thymidine-synchronized RH^{TK+} parasites was obtained for equivalent cell cycle time points in each model; R1, R3, R5 for the thymidine model and R2, R4, R6 for the PDTC model. RH strain parasites growth arrest for 6 h in the G1 phase by treatment with 60 μ M PDTC results in post-drug released populations that had larger and more variable forward scatter distributions (see R2, R4, and R6 FSC values below) as compared to RH^{TK+} parasites that are arrested at the G1/S boundary following 4 h of thymidine treatment (R1-R5, see FSC values below). New daughter parasites (with a 1N DNA content) that formed by 6 h post-release from PDTC inhibition (R6) had a smaller average FSC than R4 parasites that also contained a haploid DNA content indicating the parasites were smaller following cytokinesis. FSC values for gated 1N parasites: PDTC-treated; R2=120 average FSC, R4=162 average FSC, R6=105 average FSC. Thymidine-treated; R1=90 average FSC, R3=76 average FSC, R5=62 average FSC.

(B). Representative immunofluorescence images of asynchronously growing and PDTC-synchronized RH tachyzoites at 6 h post-release co-stained with a mouse monoclonal specific antibody for IMC1, rabbit antiserum against PCNA1, and dapi for genomic DNA. Note that in the top control panel the internal daughters forming within the vacuole of four mother parasites have consumed nearly all the mother cell but have not yet budded out of the mother cell (see phase image of control panel). In contrast, the four daughter parasites from the PDTC-synchronized culture budded out of the mother cell (phase image lower panel) and into the vacuole leaving behind substantial mother cytoplasmic mass (see arrows in lower panel). The discarded cytoplasmic mass was positively stained for IMC1, however, nuclear material as stained by dapi and antiserum against PCNA1 was completely and evenly distributed into each daughter parasite. Magnification in all photomicrographs was x 1,000.

(C). Daughter formation (grey bars) and cytoplasmic discard (black bars) into the vacuole determined by IMC1 IFA in asynchronous and PDTC-synchronized populations was evaluated for 100 vacuoles selected at random. Vacuole size was also determined and the parasite doubling calculated from these results as described in Material and Methods (linear graph). Control samples were taken at times parallel to the post-drug release samples at R0 (T+6 in the control), R4 (T+10), and R8 (T+14). We also sampled control cultures at a time equivalent to drug addition (labeled T0). During the total 14 h in culture, asynchronously growth populations completed between 2 and 3 divisions consistent with the ~6 h doubling time of this parasite strain. By contrast, PDTC-synchronized cultures divided in a stepwise fashion only once during the experiment and the large increase in the fraction of parasites containing internal daughters peaked prior to parasite division. The number of vacuoles where cytoplasmic discard was observed was a relatively small fraction in asynchronous populations (asynchronous black bars), whereas, cytoplasmic discard increased dramatically in PDTC-synchronized cultures at the time of parasite division and followed the peak of internal daughter formation (PDTC black bars). A vacuole was considered to be positive for cytoplasmic discard when the volume of discard material as detected by IMC1 fluorescence was >20% of the parasite volume.

Table 1 Short term PDTIC inhibition of parasite growth is fully reversible and leads to stepwise changes in the number of parasites per vacuole.

	Number of parasites per vacuole (%)						Average vacuole size
	1	2	4	8	16	32	
Parasites per vacuole at the start of all experiments (10 h overnight growth)	4±3.63	80±3.10	16±3.07				2.28
80 µM PDTIC							
6 h drug block	1	22	77				3.5
15 h drug block		35	64	1			3.3
6 h drug block--3 h post-drug release		6	80	14			4.4
6 h drug block--7 h post-drug release		1	35	64			6.5
40 µM PDTIC							
6 h drug block		18	79	3			3.8
15 h drug block		5	49	40			5.2
6 h drug block--3 h post-drug release		6	38	56			6.1
6 h drug block--7 h post-drug release			10	74	16		8.8
20 µM PDTIC							
6 h drug block		17	79	4			3.8
6 h drug block--3 h post-drug release		2	39	58	1		6.4
6 h drug block--7 h post-drug release			6	67	24	3	10.4
No drug							
6 h asynchronous growth		3	68	29			5.1
9 h asynchronous growth		3	18	72	7		7.7
13 h asynchronous growth			3	44	19	33	17.2
Survivability of parasites exposed to 80 µM PDTIC. Plaque counts determined at 8 days following various periods of drug exposure							
Length of drug treatment							
Plaquing efficiency (% of no drug controls)							
6 h				99.9%			
9 h				95.5 %			
12 h				93.7 %			