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3-Methyladenine blocks *Toxoplasma gondii* division prior to centrosome replication

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ABSTRACT

The apicomplexan *Toxoplasma gondii* replicates by endodyogeny, in which replicated organelles assemble into nascent daughter buds within the maternal parasite. The mechanisms governing this complex sequence are not understood. We now report that the kinase inhibitor 3-methlyadenine (3-MA) efficiently blocks *T. gondii* replication. The inhibition could not be attributed to the effects of 3-MA on mammalian phosphatidylinositol 3-kinase and host cell autophagy. Furthermore, we show that accumulation of host lysosomes around the parasitophorous vacuoles was unaffected. Most 3-MA-treated parasites failed to form daughter buds or replicate DNA, indicating arrest in G1 or early S-phase. Some 3-MA-treated parasites displayed abortive cell division, in which nuclear segregation to malformed daughter buds was incomplete or asymmetrical. Electron microscopy revealed the presence of residual body-like structures in many vacuoles, even in the absence of daughter buds. Most treated parasites had otherwise normal morphology and were able to resume replication upon drug removal. 3-MA-treated and control parasites were similar with respect to the extent of Golgi body division and apicoplast elongation; however, treated parasites rarely possessed replicated centrosomes or apicoplasts. These data are suggestive of a generalized blockade of *T. gondii* cell cycle progression at stages preceding centrosome replication, rather than arrest at a specific checkpoint. We hypothesize that 3-MA treatment triggers a cell cycle pause program that may serve to protect parasites during periods, such as subsequent to egress, when cell cycle progression might be deleterious. Elucidation of the mechanism of 3-MA inhibition may provide insight into the control of parasite growth.

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1. Introduction

Toxoplasma gondii, an apicomplexan obligate intracellular parasite, infects about one third of the human population worldwide and causes severe disease in immunocompromised individuals [1]. Following the invasion of host cells and the establishment of a parasitophorous vacuole, *Toxoplasma* replicates by a mechanism termed endodyogeny, in which two daughter buds form complete cells and subsequently emerge from the mother parasite, the small unused portion of which forms a residual body. During this process, several organelles, including the Golgi apparatus, apicoplast, centrosomes, mitochondrion and nucleus, replicate and segregate into the daughter buds, while others, such as micronemes and rhoptries, form de novo. This sequence of events has recently been elucidated by a series of time-lapse microscopy studies [2–5]. The mechanisms controlling this process, however, are as yet unknown, although the existence of control points is supported by recent studies that use either forward genetic approaches [6] or pharmacologic agents [7] to block cell cycle progression. In addition to signals propagated within the parasite, these mechanisms may also be initiated via interactions with the host cell, which provides a critical source of nutrients. Many host cell organelles, including mitochondria, endoplasmic reticulum, centrosome and endocytic vesicles become closely associated with the parasitophorous vacuole [8,9]. Due to the unique features of endodyogeny, in comparison with the mammalian cell cycle, the elucidation of the mechanisms controlling *T. gondii* cell division may be of considerable value with respect to the development of novel targets for intervention.

We have previously reported that host cell autophagy contributes to the growth of *T. gondii* [10]. We now have examined the effect of 3-methyladenine (3-MA), an inhibitor of phosphatidylinositol 3-kinase (PI3K) widely used to suppress autophagy [11], on

Abbreviations: 3-MA, 3-methyladenine; GFP, green fluorescent protein; HFF, human foreskin fibroblasts; IMC, inner membrane complex; NST, nuclear sugar transporter; PI3K, phosphatidylinositol 3-kinase; PDTC, pyrrolidone dithiocarbamate; S+T-Red, S+T^{ACP}-HcRed; YFP, yellow fluorescent protein.

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the parasite. The results of these studies demonstrate that parasite endodyogeny is highly sensitive to 3-MA, independent of effects on host cell autophagy, and suggest that the drug is likely to provide a valuable tool for the elucidation of critical early events in the *Toxoplasma* cell cycle.

2. Materials and methods

2.1. Parasites and cell culture

RH strain *T. gondii* and derived strains were maintained in human foreskin fibroblasts (HFFs). Green fluorescent protein (GFP)-expressing parasites (GFP-RH) have been described [12]. Yellow fluorescent protein (YFP)-expressing parasites (YFP-RH) [13] were a kind gift of B. Striepen (Univ. of Georgia). RH parasites expressing the apicoplast luminal marker, S+T^{ACP}-HcRed (S+T-Red) [14], or additionally expressing the apicoplast membrane protein FtsH1, tagged with V5 and HA epitopes [15], were used for analysis of the apicoplast. A cell line expressing an HA-tagged form of a nucleotide-sugar translocator (NST1) was used for analysis of the Golgi apparatus (unpublished results). In some cases the cells also expressed the Golgi marker GRASP55-YFP [16]. Fibroblast monolayers grown on coverslips were infected with the above cell lines. Host cells were cultured in DMEM (Invitrogen) containing 10% fetal bovine serum (Hyclone). Macrophages were obtained by lavage of mice injected 4 days previously with 1 ml of 3% thioglycolate broth (Difco). Cells were cultured for 1 day prior to infection with *T. gondii*. Multiplicity of infection was either 1 or 4, yielding comparable inhibitor effects. Treatments with 3-MA (Sigma), LY294002 (LC Labs) or wortmannin (EMD) were initiated 3–4 h post-infection as indicated, to permit completion of invasion and parasitophorous vacuole formation. For plaque assay, infected HFF cultures in multiwell plates were stained with crystal violet after paraformaldehyde fixation and entire wells were photographed. A set of 10 random fields (excluding the outer 20% of the well radius) was designed in ImageJ and applied to replicate wells. The value for each well was determined as the mean number of plaques/field. For knockdown of Vps34, HeLa cells were transfected with either nonspecific siRNAs (D-001810-0X, Dharmacon) or predesigned siRNA for hVps34 (GCAAACUCUUCCACAUAGAdTdT, Sigma). Cells were reseeded at 24 h post-transfection and infected on the following day with YFP-RH at a multiplicity of infection of 4. Infected cells and uninfected controls were harvested for flow cytometry and immunoblotting.

2.2. Flow cytometry

For analysis of intracellular parasite content, cells infected with GFP-RH or YFP-RH parasites were trypsinized, washed with PBS, fixed with 2% buffered paraformaldehyde, washed and analyzed by flow cytometry (FACSCalibur, Becton Dickinson). The data were analyzed with FCS Express (De Novo Software). The number of parasites per infected cell was calculated as the mean fluorescence divided by the fluorescence of single extracellular parasites in the same sample, thereby controlling for differences in GFP expression levels. A sorting analysis verified the linear relationship between fluorescence and parasite number [17].

2.3. Western blotting

Cells were lysed in RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS in PBS) supplemented with a protease inhibitor cocktail (Sigma). Protein concentration was determined by the microbicinchoninic acid assay (Pierce). Lysates (20 μg protein) were subjected to SDS-PAGE on 15% gels and transferred to nitrocellulose membranes. After blocking, the membranes were probed with anti-beclin (Novus Biologicals), anti-LC3 (a kind gift of R. Kopito), or anti-β-Actin (Abcam), followed by horseradish peroxidase-conjugated secondary antibody (KPL), and detection with enhanced chemiluminescence (ECL, Amersham). For FtsH1 immunoblots, intracellular V5-FtsH-HA-expressing parasites were treated with 10 mM 3-MA for 20 h. Lysates from 10^7 parasites were separated on 7.5% SDS-PAGE gels followed by transfer to nitrocellulose. Blots were blocked and incubated with antibodies as previously described [18]. Bound antibodies were detected using the LI-COR Odyssey.

2.4. Immunofluorescence and electron microscopy

For light microscopy, cells were seeded on coverslips in multiwell plates. Cells were stained as described [14] or using the following protocol. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. After blocking with 10% fetal bovine serum in PBS, the samples were incubated with primary antibodies diluted in 1% BSA washed and incubated with Alexa 488-, Alexa 680-, FITC- or Cy5-conjugated secondary antibodies (Invitrogen, Southern Biotechnology) for 1 h at room temperature. After extensive washing and staining with DAPI, coverslips were mounted with ProLong Gold anti-fade reagent (Invitrogen). The primary antibodies used were anti-V5 (Invitrogen), anti-LAMP1 (BD Bioscience), anti-centrin (a kind gift of J. Salisbury), and anti-

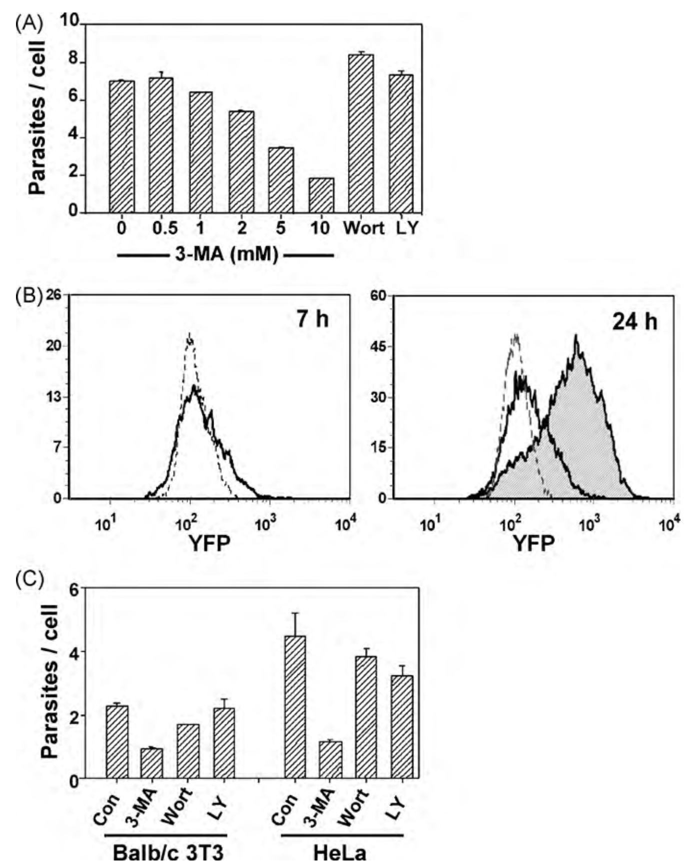

Fig. 1. 3-MA inhibits *T. gondii* proliferation. (A) HFF infected with GFP-RH were exposed to the indicated concentrations of 3-MA, or to wortmannin (100 nM) or LY294002 (10 μM). Parasites per infected cell were determined by flow cytometry (mean ± S.E., $n = 3$). (B) Flow cytometric histograms of parasite content of peritoneal macrophages infected with YFP-RH. After 4 h of infection, cultures were treated with 10 mM 3-MA and analyzed for YFP content at either 7 h or 24 h post-infection. Dotted lines display the intensity of single parasites (extracellular). At 7 h, untreated (solid line) and 3-MA-treated cells (not shown) are similar. At 24 h, 3-MA-treated cells (unshaded) showed reduced proliferation compared to control (shaded). (C) GFP-RH-infected HeLa or BALB/c 3T3 cells were treated for 20 h with 10 mM 3-MA, 100 nM wortmannin, or 10 μM LY 294002.

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