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Synthetic retinoid Am80 controls growth of intracellular *Toxoplasma* by inhibiting acquisition and synthesis of cholesterol in macrophages

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ABSTRACT

Because the obligate intracellular parasite *Toxoplasma gondii* lacks the ability to synthesize cholesterol, *T. gondii* needs to obtain cholesterol from host cells. Am80 is known to reduce the cellular cholesterol and lipid bodies in macrophages. In this study, we demonstrated that inhibition of acquisition and synthesis of cholesterol in macrophage cell line J774A.1 by a synthetic retinoid Am80 suppressed the growth of *T. gondii*. Am80 inhibited *T. gondii*-induced high levels of cellular cholesterol in J774A.1 cells while the level of cellular TAG was increased by treatment with the drug. Furthermore, the expression of LDLR was down regulated in the Am80-treated cells. Am80 reduced the expression of HMG-CoA reductase and ACAT1, and increased DGAT1 expression. These results suggest that cholesterol acquisition via LDLR and cholesterol synthesis is necessary for *T. gondii* growth though the roles of cellular TAG are still unknown. Finally, we studied the effect of Am80 *in vivo*. Mice treated with 1.0 mg/kg of Am80 ameliorated acute toxoplasmosis. Our findings support the notion that modulation of the lipid metabolism in host cells is a potential strategy for the treatment and prevention of toxoplasmosis.

Keywords: *Toxoplasma gondii*; cholesterol; triacylglycerols

INTRODUCTION

Toxoplasma gondii is an obligate intracellular parasite. *T. gondii* is capable of infecting broad range of warm blooded host, including humans (Joyson *et al.*, 2001). *T. gondii* replicates exclusively inside host cells in a specialized, non fusogenic, parasitophorous vacuole (PV) and requires high levels of selected lipids for membrane biogenesis (Mordue *et al*., 1999). *T. gondii* must scavenge cholesterol from host cells because *T. gondii* lacks the ability of cholesterol synthesis. Therefore, interference with cholesterol acquisition from the host impairs parasite growth (Coppens *et al*., 2000). In fact, previous studies have shown that sterol biosynthesis inhibitors, such as statin or HMG-CoA reductase inhibitors, impair parasite replication (Nishikawa *et al.*, 2011; Cortez *et al*., 2009).

Retinoids, which are vitamin A derivatives, regulate gene expression through the action of retinoic acid receptors (RAR) and retinoid X receptors (RXR). Both RAR and RXR belong to the nuclear hormone receptor family and consist of three subtypes: α, β and γ (Mangelsdorf *et al*., 1995). RARs induce a variety of biological activities such as control of cell growth, cell differentiation and angiogenesis (Wang *et al*., 2000; Oikawa *et al*., 1993; Goddman, 1984; Sporn *et al*., 1983). Am80, a specific RAR-α agonist, was reported to have potent immunosuppressive effect. This compound remarkably ameliorated various

immunological diseases (Seino *et al*., 2004), such as acute promyelocytic leukemia (Tobita *et al*., 1997). Am80 is known to inhibit IL-6 production in splenic mononuclear cells and reduce the severity and progression of inflammatory disease models, including 2,4-dinitrofluorobenzene-induced contact dermatitis (Niwa *et al*., 2000), collagen-induced arthritis (Nagai *et al*., 1999), and allergic encephalomyelitis (Wang *et al*., 2000). In addition, Am80 reduces scavenger receptor expression and atherosclerosis by inhibiting IL-6 (Takeda *et al*., 2006). Since uptake of oxidized low-density lipoproteins (LDL) is mediated by expression of scavenger receptor, we wondered whether *T. gondii* was sensitive to Am80. This study aimed to evaluate the effects of Am80 on *T. gondii.*

MATERIALS AND METHODS

Parasite and cell cultures

The *Toxoplasma gondii* strains, RH, PLK and its recombinants, were maintained in monkey kidney adherent fibroblasts (Vero cells) cultured in Eagle's minimum essential medium (EMEM, Sigma, St. Louis, MO) supplemented with 8% heat-inactivated fetal bovine serum (FBS). The RH strain expressing GFP were generated as previously described and are subsequently referred to as RH-GFP (Nishikawa *et al.*, 2003). For the purification of tachyzoites, parasites and host-cell debris were washed in cold PBS, and the final pellet was resuspended in cold medium and passed through a 27-gauge needle and a 5.0-μm-pore filter (Millipore, Bedford, MA). The mouse macrophage cell line J774A.1 (ATCC no. TIB 67), was cultured in Dulbecco's modified Eagle's Medium (DMEM, Sigma) supplemented with 10% heat-inactivated FBS.

Mice

C57BL/6J female mice, 6–7 weeks of age, were obtained from Clea Japan (Tokyo, Japan). Until their use at 7–8 weeks of age, mice were housed under specific pathogen-free conditions in the animal facility of the National Research Center for Protozoan Diseases at the Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan. Mice used in this study were cared for and used under the Guiding Principles for the Care and Use of Research Animals promulgated by the Obihiro University of Agriculture and Veterinary Medicine.

Reagents

Phycoerythrin (PE)-labeled anti-mouse CD11b monoclonal antibody (mAb) and purified rat anti-mouse CD16/CD32 (Fcγ III/II receptor) mAb (FcBlock^{TM}) were purchased from BD PharMingen (San Diego, CA). Anti-LDLR mouse mAb (15C8) and anti-SR-A rat mAb (2F8) were purchased from Calbiochem (Darmstadt, Germany) and Cell Sciences (Canton, MA), respectively. Alexa Fluor® 488 goat anti-mouse or rat IgG were obtained from Molecular Probes (Eugene, OR). Am80 was purchased from Wako Pure Chemical Industries (Tokyo, Japan).

RT-PCR analysis

RNA of the cells was extracted using the Trizol reagent (Gibco BRL, Grand Island, NY). Reverse transcription of 4 μg RNA was performed using Superscript II Reverse Transcriptase (Gibco BRL) in a final volume of 25 μl. PCR was performed in 50 μl of reaction mixture containing 1 μl of the reverse-transcribed RNA diluted in a buffer consisting of 0.2 mM deoxynucleoside triphosphates, 2.5 mM

$MgCl_2$, 2 μM of each primer, and 1 U *Taq* polymerase (Perkin-Elmer, Boston, MA). After initial incubation for 10 min at 95 °C, samples were subjected to cycles of denaturing at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min. After 25 cycles, the program executed a final extension of 10 min at 72 °C. The final PCR products were electrophoresed on 1.5% agarose gels and visualized using ultra violet (UV) light illumination after ethidium bromide staining. Band intensities were quantified by a density meter (Luminous Imager version 2.0, Aisin cosmos, Tokyo, Japan). Data represents the relative amount of amplified target mRNA normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the same sample. The primer sequences (sense and antisense sequences) used and the PCR product size in cDNA amplification were as follows: mouse natural cholesterol esterase (NCEase), 5' CTC CTC ATG GCT CAA CTC CTT TCC 3' and 5' AGG GGT TCT TGA CTA TGG GTG 3', 434 bp; mouse hormone sensitive lipase (HSL), 5' GCT GGT GCA GAG AGA CAC 3' and 5' GAA AGC AGC GCG CAC GCG 3', 408 bp; mouse hydroxymethylglutaryl-CoA (HMG-CoA) reductase, 5' GGG ACG GTG ACA CTT ACC ATC TGT ATG ATG 3' and 5' ATC ATC TTG GAG AGA TAA AAC TGC CA 3', 882 bp; mouse acid cholesterol esterase (ACEase), 5' GGC GGA AGA ACC ATT TTG G 3' and 5' ATT GAG AGA CAA CAC GGG AG 3', 415 bp; mouse ACAT1, 5' GGA CAA TGG TGG GTG TGC AC 3' and 5' AGA GTT CCA CCA GTC CTT AT 3', 1 kbp; mouse DGAT1, 5' CTC CTA CTT TGT GTT ATG AAC 3' and 5' GAA TCG GCC CAC AAT CCA 3', 569 bp; mouse GAPDH, 5' GAG AAC GGG AAG CTT GTC ATC AAT GG 3' and 5' ATG TGA GTC CTT CCA CGA TAC CAA AG 3', 339 bp.

Flow cytometry

After washing harvested cells (1×10^6) with cold PBS, the cells were suspended in cold PBS containing 0.5% bovine serum albumin treated with FcBlock™ to avoid non-specific adherence of mAb to Fc receptors and subsequently incubated with primary antibodies, followed by Alexa Fluor® 488-conjugated secondary antibody. Labeled cells (1×10^4) were examined using an EPICS ® XL flow cytometer (Beckman Coulter, Hialeah, FL). For analysis of cells from the peritoneal cavity, macrophages were gated as the $CD11b^+$ cells.

Growth analyses of parasite and host cell

J774A.1 cells (1×10^5) infected with *T. gondii* tachyzoites (1×10^5) were cultured with 0.5 ml medium in 24-well plates. After incubation for 20 hours at 37 °C, [5,6-^{3}H] uracil (Moravek Biochemicals, Brea, CA) was added to the plate at 1 μCi/well, and the cell mixtures were further incubated for 2 hours at 37 °C. After fixation with 10% trichloroacetic acid, the cell mixtures were incubated with 0.2 N NaOH for 30 min at 37 °C. The radioactivity incorporated into the parasites was measured by counting the radioactivity using a beta counter. To measure the growth of J774A.1 cell, the cells were plated on 96-well microplates at 2.5 × 10^4/well. After incubation of the 96-well microplates for 48 hours at 37°C, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt and 1-methoxy-5-methylphenazinium methylsulfate (Cell Counting Kit-8, Dojin Laboratories, Japan) were added to final concentrations of 5 mM and 0.2 mM respectively and the mixture was further incubated for 1 hour. The results of proliferation were expressed as the mean absorbance at 450 nm. Cell viability represents the relative value of drug-treated cells normalized against untreated cells (×100).

Treatment of *T. gondii*-infected mice with Am80

Mice were injected i.p. with or without Am80 in 0.25 ml of sterile, endotoxin-free EMEM, on day 1 before infection i.p. with 1×10^3 *T. gondii* tachyzoites of PLK strain. After parasite infection, the drugs were injected daily for 7 consecutive days, starting at the time of *T. gondii* infection. All control mice were injected in the same manner with 0.25 ml of EMEM.

RESULTS

Am80 has anti-*T. gondii* effects

At first, we examined the effect Am80 on cultured parasite in J774A.1 cells. Although Am80 did not affect the parasite infection rates (Fig. 1A), this drug decreased vacuole size (Fig. 1B). We also confirmed the anti-toxoplasma effects of Am80 by uracil incorporation assay (Fig. 1C). The toxicity towards the J774A.1 cells was not observed below 100 μM (data not shown) while the growth of the host cells slightly reduced at 50 μM Am80.

Am80 inhibits the acquisition and synthesis of cholesterol by *T. gondii*

To further analyze effects of Am80 on *T. gondii* growth, the levels of intracellular cholesterol and triacylglycerol (TAG), and expression of LDLR in Am80-treated J774A.1 cells were measured. Am80 dose-dependently reduced the amounts of intracellular cholesterol, indicating the importance of cellular cholesterol for *T. gondii* growth (Fig. 2A). On the other hand, intracellular TAG was increased by Am80 (Fig. 2A). These results suggest that the intracellular TAG did not support *T. gondii* growth under the low levels of intracellular cholesterol in J774A.1 cells. The levels of LDLR expression were reduced by Am80 (Fig. 2B). On the other hand, treatment of the cells with Am80 enhanced the levels of SA-R expression (Fig. 2B). These results indicated that Am80 suppressed the *T. gondii* growth in macrophages by inhibiting LDLR expression and that cholesterol acquisition via LDLR was important for parasite growth. These observations were consistent with the results of RT-PCR of Am80-treated cells (Fig. 3). Treatment of the infected and uninfected J774A.1 cells with Am80 showed downregulation of ACAT1 and upregulation of DGAT1. These expression patterns might result in low levels of cellular cholesterol and high levels of TAG caused by Am80 treatment. Interestingly, levels of HMG-CoA reductase expression were also decreased by Am80. Collectively, the results suggest that Am80 could control the acquisition and synthesis of cholesterol.

Effects of Am80 on toxoplasmosis in mouse model

Given our observations that Am80 effectively blocked *T. gondii* growth *in vitro*, we hypothesized whether this drug might control toxoplasmosis *in vivo*. To test this idea, BALB/c mice (n = 6) infected with *T. gondii* tachyzoites were treated with Am80 (Fig. 4A). Compared with control animals, the lifetime was prolonged in mice treated with 1.0 mg/kg of Am80 (Fig. 4A). To confirm the effects of 1.0 mg/kg of Am80, animal numbers for the test were increased (n=18). Percent survival of mice treated with 1.0 mg/kg of Am80 increased statistically (Fig. 4B). Median survival was 15 days and 19 days in control mice and treated mice, respectively. In addition, speed until death in control mice was 1.945 times faster than in the treated mice. This result indicates that Am80 treatment could control acute *T. gondii* infection.

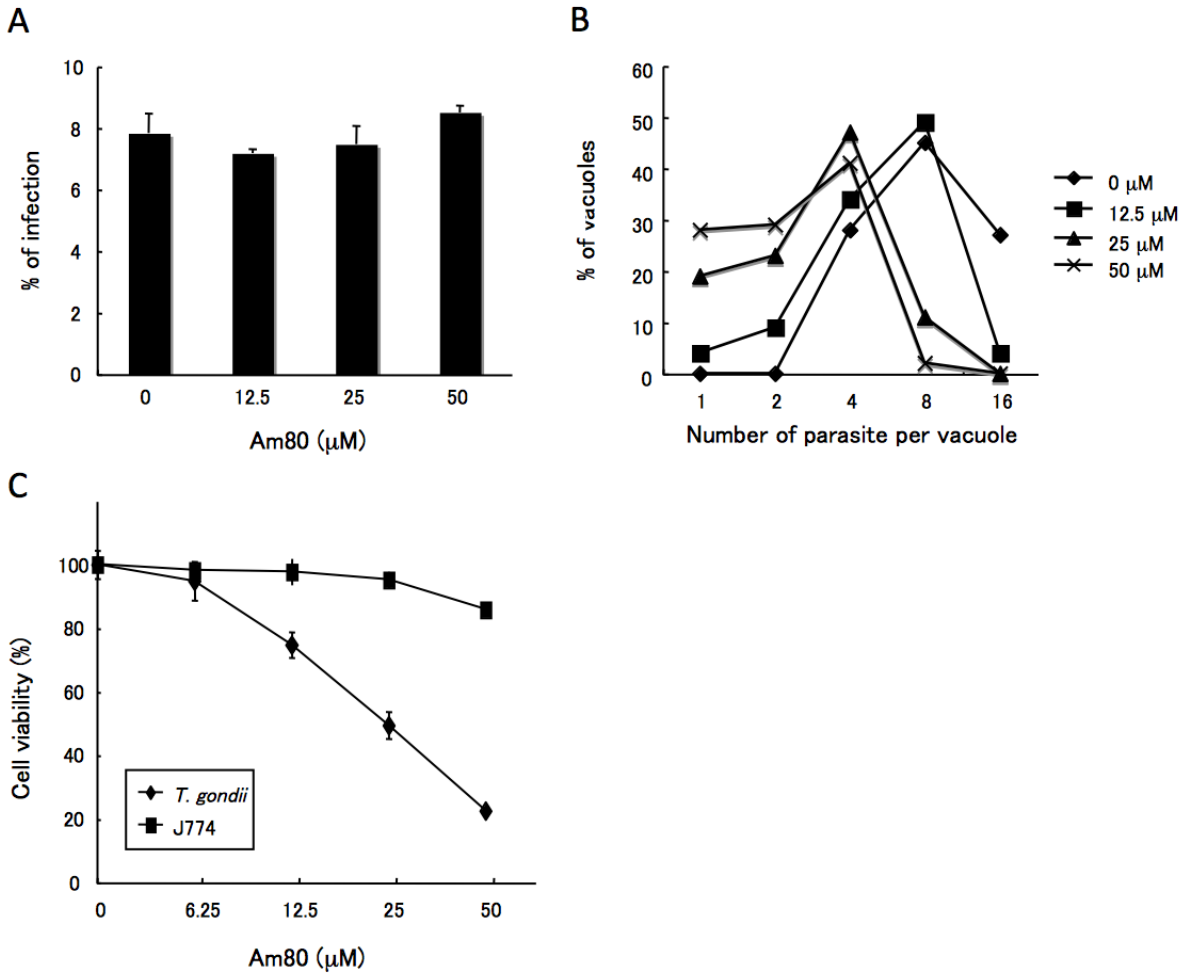

Fig. 1. Effects of Am80 on *T. gondii* growth.

(A) J774A.1 cells were pretreated with Am80 for 6 hours, and then infected with RH-GFP (MOI of 0.5) for an additional 20 hours in the presence of Am80. Infection rates of the parasite were examined by flow cytometry to detect the GFP-positive J774A.1 cells. Each value represents the mean ± standard deviation of triplicate samples. Statistical analysis of the data was carried out using 1-way ANOVA followed by Tukey's multiple comparison test. (B) J774A.1 cells were pretreated withAm80 for 6 hours, and then infected with *T. gondii* (MOI of 1) for an additional 40 hours in the presence of Am80. The harvested cells were stained with anti-SAG1 mAb, followed by Alexa Fluor® 488 goat anti-mouse IgG to detect the tachyzoites in J774A.1 cells. The distribution of PV size expressed in percent (%) was determined. Data were determined by 100 randomly selected vacuoles. (C) J774A.1 cells were pretreated with Am80 for 6 hours, and then infected with or without *T. gondii* (MOI of 1) for an additional 20 hours in the presence of Am80. Cell viabilities were calculated as described in Materials and Methods. Each value represents the mean ± standard deviation of quadruplicate samples.

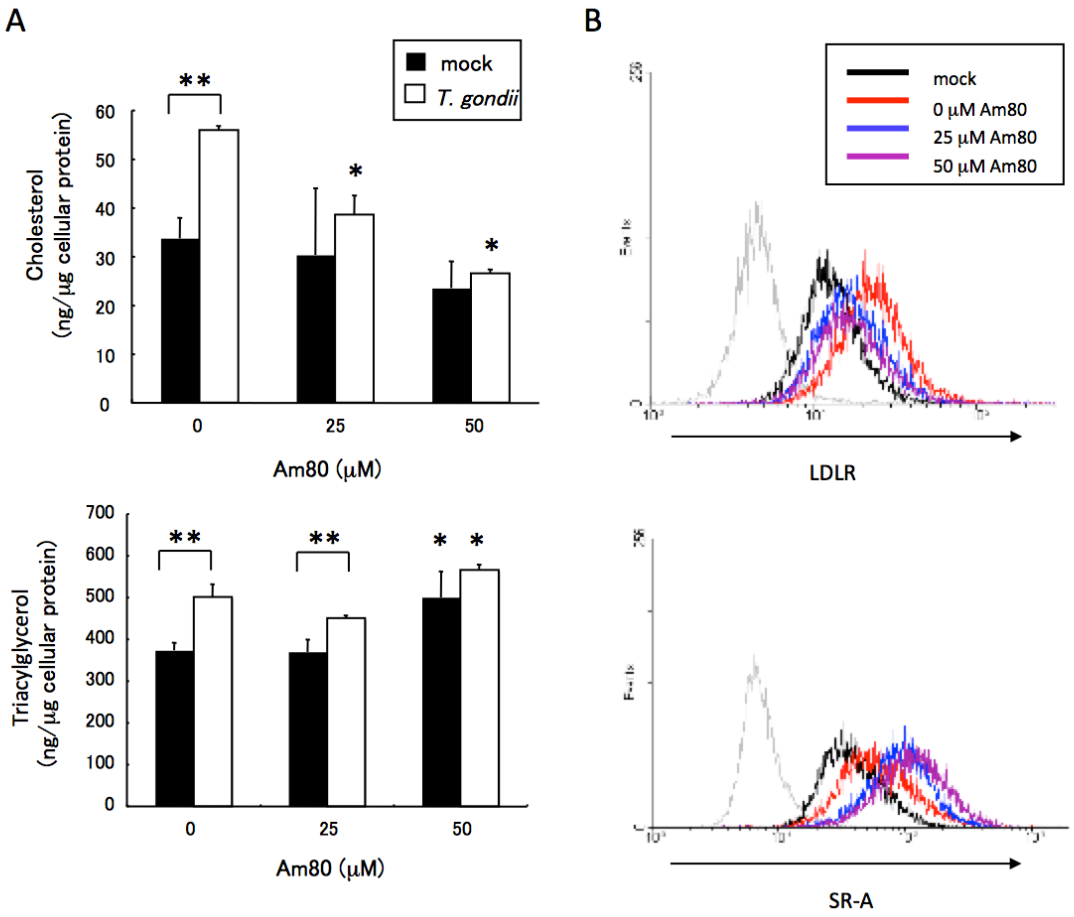

Fig. 2. (A) Effects of Am80 on cellular cholesterol and TAG. J774A.1 cells were pretreated with Am80 for 6 hours, and then infected with *T. gondii* (MOI of 1) for an additional 40 hours in the presence of Am80. Intracellular cholesterol and TAG were measured as described in Materials and Methods. Statistical analysis of the data was carried out using Student's *t*-test. (*) Values of $P<0.05$ were considered significant between the mock- and *T. gondii*-infected cells at same dose of Am80. (**) Values of $P<0.05$ were considered significant compared with the treatment of mock- or *T. gondii*-infected cells with 0 μM Am80. (B) Effects of Am80 on expression of LDLR and SR-A in J774A.1 cells. J774A.1 cells were pretreated with Am80 for 6 hours, and then infected with *T. gondii* (MOI of 1) for an additional 40 hours in the presence of Am80. Expression of the receptors was examined by flow cytometry using anti-LDLR mAb and SR-A mAb.

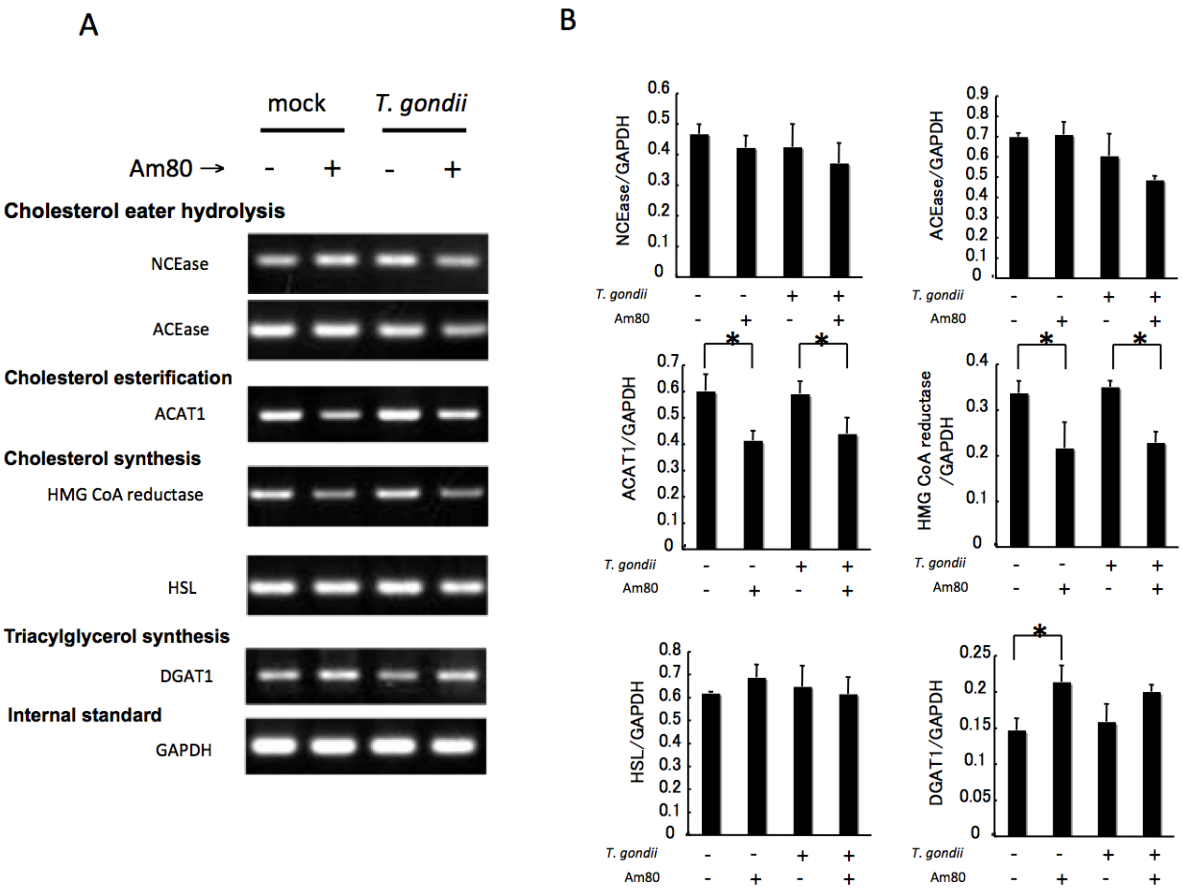

Fig. 3. RT-PCR analyses for J774A.1 treated with Am80. (A) J774A.1 cells were pretreated with Am80 (50 μM) for 6 hours, and then infected with *T. gondii* (MOI of 1) for an additional 20 hours in the presence of Am80. The total RNA was isolated from parasitized or mock-infected J774A.1 and used for RT-PCR. (B) Band intensities were quantified by densitometry. Bars represent the relative amount of amplified target mRNA against GAPDH mRNA in the same sample. Each value represents the mean of the target mRNA/GAPDH mRNA ± the standard deviation of triplicate samples. Statistical analysis of the data was carried out using 1-way ANOVA followed by Tukey's multiple comparison test. (*) Values of $P<0.05$ were considered significant.

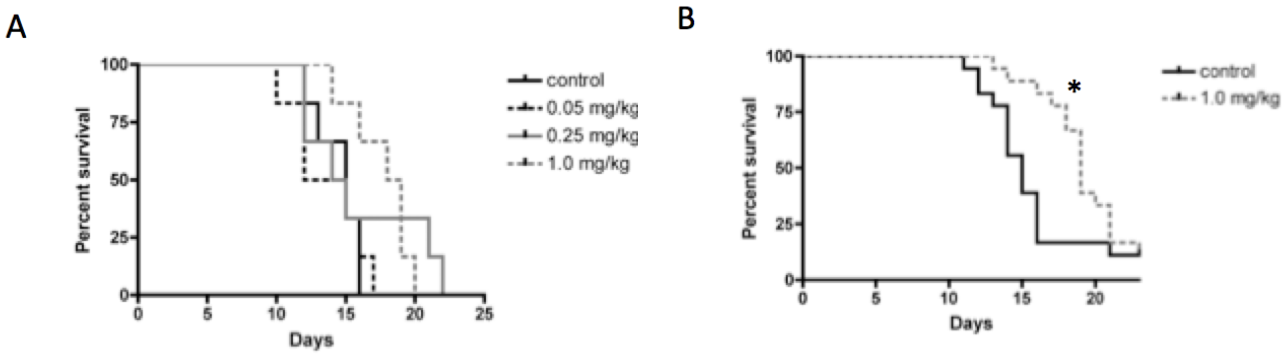

Fig. 4. Surval of mice following acute challenge with *T. gondii* and treatment with Am80. (A) BALB/c mice (n = 6) were treated with several doses of Am80 on day 1 before infection i.p. with 1×10^3 *T. gondii* tachyzoites of PLK strain. After parasite infection, the drugs were injected daily for 7 consecutive days, starting at the time of *T. gondii* infection. (B) Treatment of mice with Am80 (1.0 mg/kg) was repeated. Data were combined from three independent experiments (n = 18). Statistical analysis of the data was carried out using the method of Kaplan-Meier followed by log-rank test. (*) Values of $P<0.05$ were considered significant.

DISCUSSION

T. gondii must obtain sterol from host cells because *T. gondii* lacks the ability to synthesis them via the mevalonate pathway, (Coppens *et al*., 2000). There are three mechanisms that host exploit to supplement depleted cholesterol stores: 1) by promoting *de novo* cholesterol synthesis via the mevalonate pathway; 2) by activating uptake of cholesterol; or 3) recruitment of intracellular CE. *De novo* cholesterol synthesis relies on the mevalonate pathway. HMG-CoA reductase is a rate-limiting enzyme of the mevalonate pathway. The levels of HMG-CoA reductase expression were increased in macrophages 20 hour after *T. gondii* infection (Kameyama *et al*., 2011). A similar result by microarray analysis was observed in human foreskin fibroblasts infected with *T. gondii* (Blader *et al*., 2001). Moreover, for activated LDL uptake, levels of LDLR expression were upregulated upon *T. gondii* infection at 40 hours post-infection (Kameyama *et al*., 2011). Together, synthesis and uptake of host cholesterol are important for *T. gondii* growth.

At first, we expected that Am80 suppressed the function of scavenger receptor. However Am80 had anti-toxoplasma effects while this drug did not inhibit the expression of scavenger receptor. In addition, levels of intracellular cholesterol were suppressed by Am80 in J774A.1 cells infected with *T. gondii* because of lower levels of LDLR expression. On the other hand, levels of intracellular TAG were increased by Am80, suggesting a low contribution of intracellular TAG for parasite growth though the effects of intracellular TAG on *T. gondii* growth are still unknown. Thus, Am80 inhibited parasite growth by decreasing intracellular accumulation of cholesterol. Then we examined how Am80 regulated expression of genes, which related to cholesterol metabolism in macrophage. Am80 downregulated both HMG-CoA reductase and ACAT1 expression, indicating the lower level of cellular cholesterol. To maintain the lipid content in host cells treated with Am80, DGAT1 expression might be increased. The low level of cellular cholesterol could affect *T. gondii* replication because the parasite membranes and rhoptries contain

cholesterol derived from host cells (Coppens *et al*., 2003). Although our study showed that Am80 modulated lipid metabolism to inhibit *T. gondii* replication, but not the invasion, the relationship between the parasite and host retinoic acid receptor of host cells is less clear.

Inhibiting cholesterol acquisition by interfering with the LDL endocytic pathway of the host reduces parasite replication, indicating that it might be a realistic strategy for therapeutic purposes. However, the possibility of cytotoxicity of Am80 remains to be seen. Our study shows that Am80 had more growth-inhibition effects against *T. gondii* than host cells *in vitro*. Moreover, Am80 could ameliorate the acute toxoplasmosis in mouse models. In conclusion, our findings support the notion that modulation of lipid metabolism in host cells is a potential strategy for the treatment and prevention of toxoplasmosis.

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