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Short communication

In vitro and *in vivo* effects of the phytohormone inhibitor fluridone against *Neospora caninum* infection

Rochelle Haidee D. Ybañez [a,b,1], Arpron Leesombun [a,1], Maki Nishimura [a], Ryuma Matsubara [c,d], Mikiko Kojima [e], Hitoshi Sakakibara [e], Kisaburo Nagamune [c,f], Yoshifumi Nishikawa [a,*]

[a] National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan
[b] Biology and Environmental Studies Program, University of the Philippines Cebu, Gorordo Avenue, Lahug, Cebu City 6000, Philippines
[c] Department of Parasitology, National Institute of Infectious Diseases, Toyama, Shinjuku-ku, Tokyo 162-8640, Japan
[d] Graduate School of Life and Environmental Studies, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan
[e] RIKEN Center for Sustainable Resource Science, 1-7-22, Suehiro, Tsurumi, Yokohama 230-0045, Japan
[f] Faculty of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan

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ABSTRACT

Neospora caninum causes abortion and stillbirth in cattle. Identification of effective drugs against this parasite remains a challenge. Previous studies have suggested that disruption of abscisic acid (ABA)-mediated signaling in apicomplexan parasites such as *Toxoplasma gondii* offers a new drug target. In this study, the ABA inhibitor, fluridone (FLU), was evaluated for its action against *N. caninum*. Production of endogenous ABA within *N. caninum* was confirmed by ultra-performance liquid chromatography–tandem quadruple mass spectrometry. Subsequently, FLU treatment efficacy was assessed using *in vitro*. Results revealed that FLU inhibited the growth of *N. caninum* and *T. gondii in vitro* (IC_{50} 143.1 ± 43.96 μM and 330.6 ± 52.38 μM, respectively). However, FLU did not affect parasite replication at 24 h post-infection, but inhibited egress of *N. caninum* thereafter. To evaluate the effect of FLU *in vivo*, *N. caninum*-infected mice were treated with FLU for 15 days. FLU treatment appeared to ameliorate acute neosporosis induced by lethal parasite challenge. Together, our data shows that ABA might control egress in *N. caninum*. Therefore, FLU has potential as a candidate drug for the treatment of acute neosporosis.
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Neospora caninum, the causative agent of bovine neosporosis, is an obligate, intracellular, apicomplexan parasite that is genetically different from, but structurally similar to, *Toxoplasma gondii* [1]. The disease it causes is closely associated with abortion and stillbirth in the dairy industry [1]. Recently, assessment of the global economic impact of *N. caninum* revealed annual losses ranging from 1.1 million USD in New Zealand to more than 1 billion USD in the USA, with more losses occurring in the dairy industry than in the beef industry [2]. Control and treatment of this parasitic disease is important, but efforts have thus far been limited. Drugs such as sulfonamides, clindamycin, pyrimethamine, and ponazuril are available for treatment of canine neosporosis [3]. Nevertheless, development of new drug treatment options for *N. caninum* remains a challenge.

It was recently discovered that the closely related protozoan parasite *T. gondii* produces and uses the plant hormone, abscisic acid (ABA) [4]. ABA is an important phytohormone that regulates plant growth, development, dormancy, and stress responses [5]. In a recent study using fluridone (FLU), which is an inhibitor of ABA synthesis, ABA was shown to control the calcium-dependent egress and development of *T. gondii* [4]. Calcium signaling is a very important pathway that regulates diverse cellular processes [6]. In apicomplexan parasites, this signaling pathway directs motility, cell invasion, and egress [7]. Increase in the concentration of ABA causes calcium ion influx, thereby triggering exit of the parasite from infected cells. This process occurs in the apicoplast, a plant-like derivative organelle that is a plastid homolog in plants [4]. *N. caninum* invades a host cell during its tachyzoite stage, replicates within a parasitophorous vacuole (PV), and lyses the host cell during egress to start another cycle of infection. Although the role of calcium signaling in *N. caninum* is unknown, addition of the calcium ionophore A23187 was effective at releasing the parasite from its host cells [8]. In common with all apicomplexan parasites, *N. caninum* contains an apicoplast [9]. Thus, these results suggest that calcium signaling may play a crucial role in *N. caninum* egress.

FLU is generally used as an herbicide, but because it has an effect on the apicoplast, it could be a potential drug against *N. caninum*. Thus, studying the action of FLU against *N. caninum* is worthwhile. The present study aimed to evaluate the *in vitro* and *in vivo* actions of FLU against *N. caninum* infection.

* Corresponding author at: National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan.
E-mail address: nisikawa@obihiro.ac.jp (Y. Nishikawa).
[1] Both authors contributed equally to this work.

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N. caninum-expressing green-fluorescent protein (Nc-GFP) [10], *N. caninum* (Nc-1 strain), GFP-expressing *T. gondii* (RH-GFP strain) [11] and *T. gondii* (RH strain) were propagated and maintained using monolayers of African green monkey kidney (Vero) cells in Eagle's minimum essential medium (MEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 8% heat-inactivated fetal bovine serum. Purification of the tachyzoites was done by washing the parasites with the medium to remove host-cell debris. The parasite pellet was resuspended in the same medium and passed through a 27-gauge needle and a MF-Millipore 5.0-μm-pore membrane filter (Millipore, Bedford, MA, USA).

To confirm that ABA is produced in *N. caninum* (Nc-1 strain) and to compare it with *T. gondii* (RH strain), the hormone was extracted from purified tachyzoites of both parasites using ultra-performance liquid chromatography (UPLC)–tandem mass spectrometry (AQUITY UPLC System/XEVO-TQS; Waters, Milford, MA, USA) with an ODS column (AQUITY UPLC BEH C18, 1.7 μm, 2.1 × 100 mm; Waters) as described previously [12].

Monolayers of human foreskin fibroblast (HFF) cells were grown in 96-well plates (cell suspensions 1×10^5 cells/ml in DMEM supplemented with 10% FBS). To examine the effects of FLU on the intracellular parasites, RH-GFP (5×10^4 tachyzoites per well) was added to the wells for 4 h and the extracellular parasites were washed away. Then, FLU (Sigma-Aldrich) at the indicated concentrations (100 μl/well of medium) were added for 72 h. The fluorescence intensity of GFP was measured using a microplate reader (SH-900, Corona Electric Co., Ltd., Ibaraki, Japan). The correlation coefficient between the fluorescence intensity of GFP and the number of parasites (a two-time serial dilution ranging from 1,000,000 to 7812.5 parasites) was calculated using the Pearson correlation coefficient and a positive correlation was confirmed. The growth inhibition of GFP-expressing parasite (%) was expressed as follows: [(average fluorescence intensity of GFP with medium alone) − (the fluorescence intensity of GFP treated with FLU)/(average fluorescence intensity of GFP with medium alone)] × 100.

To measure *N. caninum* replication in HFF cells, Nc-GFP parasites grown in HFF cells were cultured on glass coverslips. Three hours after infection, the cells were treated with either FLU at 100 μM or DMSO (control). Following treatment at time intervals of 24, 48, and 72 h post-infection (hpi), parasite replication was evaluated by counting the number of intracellular parasites based on GFP signal per PV (a total of 100 randomly selected vacuoles) using an All-in-one Fluorescence Microscope (BZ-9000, Keyence, Tokyo, Japan). To confirm the parasite egress, the number of extracellular tachyzoites at 72 hpi was counted by a hemocytometer. To evaluate an impact of FLU on host cell invasion of *N. caninum*, Nc-GFP parasites were pretreated with FLU for 1 h at 37 °C. Then, the pretreated parasites were added to Vero cells (parasites per host cell ratio = 2:1) and the extracellular parasites were washed away at 2–3 hpi. The infection rates at 24 hpi were calculated by counting the number of infected cells based on GFP signal in the Vero cells (a total of 100 randomly selected Vero cells labeled with Hoechst 33342) using the Fluorescence Microscope.

Female BALB/c mice were obtained from CLEA Japan (Tokyo, Japan). Animals were housed under specific pathogen-free conditions in the animal facility at the National Research Center for Protozoan Diseases (Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan). This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Obihiro University of Agriculture and Veterinary Medicine. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Obihiro University of Agriculture and Veterinary Medicine (Permit number 24-17, 25-66). *In vivo* infections in female BALB/c mice

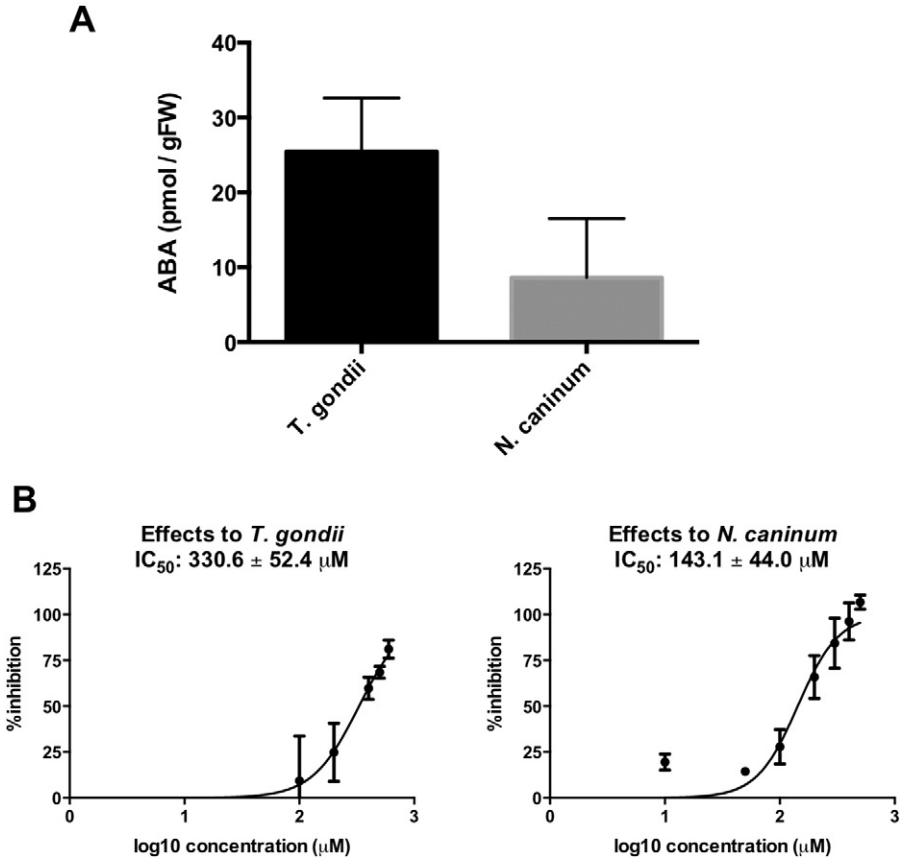

Fig. 1. (A) Quantification of ABA from *N. caninum* (Nc-1) or *T. gondii* (RH) using an ultra-performance liquid chromatography system coupled to a tandem quadrupole mass spectrometer equipped with an electrospray interface. Results are the means ± SD of three independent experiments. Student's *t*-test; $P = 0.052$. (B) Growth inhibition of *N. caninum* and *T. gondii* in parasite-infected cells treated with fluridone (FLU) at varying concentrations. Results are the means ± SD of three independent experiments performed using quadruplicate samples.

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