Veterinary Parasitology 265 (2019) 19–23

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Research paper

Inhibitory effects of the phytohormone inhibitors fluridone and inabenfide against *Babesia gibsoni in vitro*

Mingming Liu[a], Tatsunori Masatani[b,*], Paul Franck Adjou Moumouni[a], Seung-Hun Lee[a], Eloiza May Galon[a], Yang Gao[a], Huanping Guo[a], Jixu Li[a], Yongchang Li[a], Xuenan Xuan[a,*]

[a] National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido, 080-8555, Japan
[b] Transboundary Animal Diseases Research Center, Joint Faculty of Veterinary Medicine, Kagoshima University, 1-21-24 Korimoto, Kagoshima, 890-0065, Japan

ARTICLE INFO

Keywords:
Babesia gibsoni
Phytohormone
Fluridone
Inabenfide

ABSTRACT

Pharmacological options to treat canine babesiosis caused by *Babesia gibsoni*, are limited. To address this challenge, screening for novel drug candidates and drug targets against *B. gibsoni* is urgently needed. In this study, we explored the inhibitory effects of two phytohormone inhibitors, fluridone (FLU) and inabenfide (INA), against *B. gibsoni in vitro*. The half-maximal inhibitory concentration (IC_{50}) values of FLU and INA against *B. gibsoni* were 60.6 ± 3.4 and 4.3 ± 0.3 μM, respectively. Parasitemia and viability at 24, 48, and 72 h after FLU and INA treatments were significantly lower than those in the control group. The cytotoxicity of FLU and INA was evaluated using the dog-derived Madin-Darby canine kidney (MDCK) cell line; both FLU and INA were less toxic to the MDCK cells than to the control cells. The selectivity index of FLU and INA were higher than 16.5 and 232.6, respectively. In summary, the present study demonstrated that FLU and INA were effective against *B. gibsoni* infection *in vitro* and that these compounds might have potential as candidate drugs for the treatment of *B. gibsoni*.

1. Introduction

Babesia gibsoni is one of the tick-borne intraerythrocytic apicomplexan parasite that can cause canine babesiosis (Solano-Gallego and Baneth, 2011). *B. gibsoni* infection is endemic to many regions of the Americas, and Asia including Japan (Kjemtrup et al., 2000; El-Dakhly et al., 2015). Infected dogs show progressive anemia, remittent fever, hemoglobinuria, marked splenomegaly, hepatomegaly and sometimes death (Kumagai et al., 2016). *B. gibsoni* has a global distribution, with a considerable impact on canine health (Baneth, 2018). However, effective control and eventual eradication of canine babesiosis remain a huge challenge. Drugs such as atovaquone, azithromycin, clindamycin, diminazene aceturate and imidocarb dipropionate are available for the treatment of canine babesiosis due to *B. gibsoni* (Goo and Xuan, 2014; Baneth, 2018). Nevertheless, because of the strong side effects and variable effectiveness of these drugs against the parasites, development of new drug treatment options for *B. gibsoni* is needed.

Apicomplexan parasites, including *Babesia* spp., have been considered to be plant-like organisms by some authors (McFadden and Yeh, 2017). One reason is that these parasites possess an apicoplast, a remnant organelle of secondary endosymbiosis of algal origin. The apicoplast has features similar to those of plastids in plants and involved in the biosynthesis of heme, fatty acids, iron-sulfur clusters, and isoprenoids in the parasite (Lim and McFadden, 2010; Sato, 2011; Arisue and Hashimoto, 2015). In addition, apicomplexan parasites produce phytohormones, such as abscisic acid (ABA) (Nagamune et al., 2008a; Ybañez et al., 2016), gibberellins (GA) (Toyama et al., 2012), salicylic acid (Matsubara et al., 2015), and cytokinins (Andrabi et al., 2018). Previous studies (Nagamune et al., 2008a; Matsubara et al., 2015; Ybañez et al., 2016) have revealed that phytohormones are essential for the infectivity of apicomplexan parasites including *Toxoplasma gondii*, *Neospora caninum*, and *Plasmodium* spp., and phytohormone inhibitors have been extensively studied for their anti-apicomplexan effects.

ABA is an important phytohormone that regulates stress responses, seed dormancy, and embryo development (Shu et al., 2018). Recent studies have reported that fluridone (FLU) is an inhibitor of ABA synthesis, which has been shown to affect the calcium-dependent release and development of *T. gondii*, and inhibit the release of *N. caninum* from infected cells (Nagamune et al., 2008b; Ybañez et al., 2016). GA, another essential phytohormone, regulates various developmental processes, including stem elongation, germination, dormancy,

* Corresponding authors.
E-mail addresses: masatani@vet.kagoshima-u.ac.jp (T. Masatani), gen@obihiro.ac.jp (X. Xuan).

https://doi.org/10.1016/j.vetpar.2018.11.018
Received 27 September 2018; Received in revised form 2 November 2018; Accepted 23 November 2018
0304-4017/ © 2018 Elsevier B.V. All rights reserved.

flowering, flower development, and even leaf and fruit senescence (Gupta and Chakrabarty, 2013). A previous study indicated that inabenfide (INA) is an inhibitor of GA synthesis, and exerted strong effects in both *Plasmodium falciparum* and *T. gondii* (Toyama et al., 2012). Therefore, FLU and INA may have potential as candidate drugs for the treatment of diseases caused by apicomplexan parasites. However, there are no reports regarding the production of phytohormones in *Babesia* parasites. Thus, this study aimed to evaluate the inhibitory effects of the phytohormone inhibitors FLU and INA on the growth and replication of *B. gibsoni in vitro*.

2. Materials and methods

2.1. Parasite and cell culture

Wild-type *B. gibsoni* Oita strain (Bg-WT) (Sunaga et al., 2002) and green fluorescent protein-expressing *B. gibsoni* Oita strain (Bg-GFP) (Liu et al., 2018) were cultured *in vitro* in 24-well culture plates (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C in an incubator (BIO-LABO; Juji-Field, Tokyo, Japan) with a humidified atmosphere (5% CO_2 and 5% O_2). The parasites were cultured in 10% canine erythrocytes suspended in RPMI-1640 supplemented with 20% canine serum.

Madin-Darby canine kidney (MDCK) cells were maintained in a 75-cm^2 culture flask containing Minimum Essential Medium (MEM; Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin (Gibco, Life Technologies, USA), 0.15% $NaHCO_3$ (Wako, Osaka, Japan), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at a final concentration at 25 mM and cultured at 37 °C in an incubator with a humidified atmosphere (5% CO_2 and 5% O_2). The medium was changed every 2 days and incubated until approximately 80% confluence was reached.

2.2. Reagents

FLU (Sigma-Aldrich, Tokyo, Japan), INA (Wako, Osaka, Japan) and diminazene aceturate (DA, Sigma-Aldrich, Tokyo, Japan) were dissolved in dimethyl sulfoxide (DMSO, Wako, Osaka, Japan) to prepare a 100 mM stock solution and stored at −30 °C.

2.3. Inhibition assay of FLU, INA, and DA in vitro

The experiment was conducted on 1% parasitemia at 2.5% hematocrit by using Bg-WT for four consecutive days without changing the media. The final concentrations of FLU and INA in the cultures ranged from 5 to 1000 μM and 1 to 25 μM, respectively. The final concentrations of DA in the positive control wells ranged from 0.1 to 50 nM; 0.5% DMSO was used as control. Each concentration was determined in triplicate in three separate wells. Parasitemia was monitored on day 4 by examining 3,000 RBCs by using Giemsa staining.

2.4. Parasitemia in FLU- and INA-treated cells

The experiment was conducted on 1% parasitemia at 10% hematocrit by using Bg-WT in cultures containing final concentrations of 2 × half-maximal inhibitory concentration (IC_{50}), which were 121.2 μM and 8.6 μM FLU and INA, respectively. The parasitemia was monitored by examining 3,000 RBCs with Giemsa staining at 24, 48, and 72 h after FLU and INA treatments. DMSO (0.5%) was used as control.

2.5. Fluorescent microscope images and viability of FLU- and INA-treated cells

The experiment was conducted on 1% parasitemia at 10% hematocrit by using Bg-GFP in cultures containing final concentrations of 2 × IC_{50}, which were 121.2 μM and 8.6 μM FLU and INA, respectively. The micrographs of Bg-GFP were obtained at 24 h after FLU and INA treatment, and viability was detected at 24, 48, and 72 h after FLU and INA treatment. Hoechst 33342 (Sigma-Aldrich, Tokyo, Japan) was used to stain the nuclei of both live and dead parasites, and propidium iodide (PI, Sigma-Aldrich, Tokyo, Japan) was used to stain only the dead parasites' nuclei. DMSO (0.5%) was used as control. Cells treated in the manner described above were examined under a confocal laser-scanning microscope TCS-SP5 (Leica, Wetzlar, Germany).

2.6. Cytotoxicity assay of FLU and INA using the MDCK cell line

The drug-exposure viability assay was performed in accordance with the recommendation provided with the Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). Briefly, MDCK cells at a density of 5×10^4 cells/ml were seeded on 0.1 ml per well of 96-well cell culture plate and allowed to attach to the plate for 24 h. One hundred microliters of twofold drug dilutions was added to each well to a final concentration of 2.5–1000 μM in triplicate. The cells were exposed to the drugs for 24 h, after which 10 μl of CCK-8 was added. The plate was further incubated for 3 h, and absorbance was measured at 450 nm by using an MTP-500 microplate reader (Corona Electric, Ibaraki, Japan). The wells with only the culture medium were used as blanks, while those containing cells in a medium with 0.5% DMSO were used as controls.

2.7. Statistical analysis

The IC_{50} values of FLU, INA, and DA were determined using the non-linear regression analysis (curve fit) in GraphPad Prism (GraphPad Software Inc., California, USA). The differences in parasitemia and parasite viability were determined using Student's *t*-test; differences were considered significant at $^*p < 0.05$.

3. Results

3.1. Growth inhibitory effects

Both FLU and INA significantly ($^*p < 0.05$) inhibited the growth of *B. gibsoni* in a dose-dependent manner. The IC_{50} values of FLU and INA were 60.6 ± 3.4 and 4.3 ± 0.3 μM, respectively (Fig. 1). The IC_{50} of DA was 3.0 ± 0.3 nM (data not shown).

3.2. Parasitemia and viability of FLU- and INA-treated cells

Parasitemia was monitored using Giemsa-stained blood smears prepared from the concentration at 2 × IC_{50}. The parasitemia at 24, 48, and 72 h after FLU and INA treatments was significantly lower ($^*p < 0.05$) than that in the control group (Fig. 2). Next, the viability of parasites treated with FLU and INA was determined by Hoechst 33342/PI double staining at 2 × IC_{50}. The viability of FLU- and INA-treated cells at 24, 48, and 72 h was also significantly lower ($^*p < 0.05$) than that in the control group (Fig. 3).

3.3. Fluorescent microscope images of FLU- and INA-treated cells

The fluorescent micrographs of Bg-GFP were obtained at 24 h after FLU and INA treatments. Both FLU and INA significantly inhibited the growth and multiplication of the parasite at 2 × IC_{50}. Dying parasites were stained by PI and showed a decreasing GFP level until the color disappeared (Fig. 4).

3.4. Cytotoxicity of FLU and INA on MDCK cell line

The potential effects of FLU and INA on the canine host were evaluated using the MDCK cell line, derived from the natural host of *B. gibsoni*. The IC_{50} values of both FLU and INA were higher than 1000 μM (Fig. 5). The selectivity index (SI), defined as the ratio of the cell-line

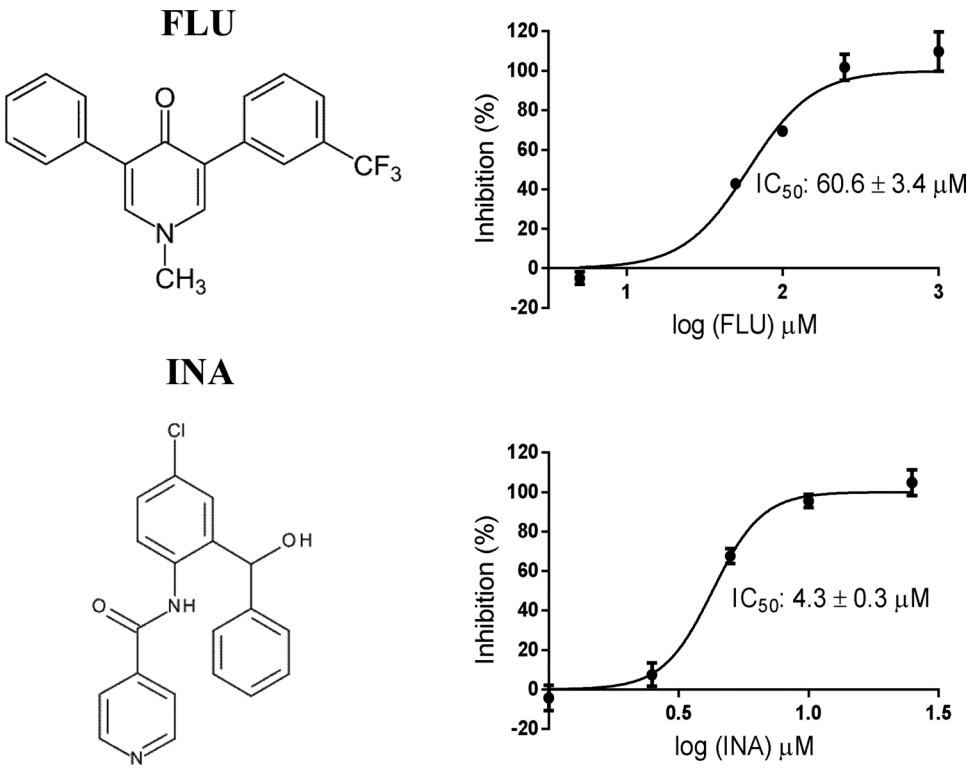

Fig. 1. Structures and dose response curves of FLU and INA against *B. gibsoni in vitro*. The experiment was conducted on 1% parasitemia at 2.5% hematocrit for four consecutive days without changing the media. DMSO (0.5%)-treated cells were used as the control group. The values are presented as mean ± S.D. of three independent experiments.

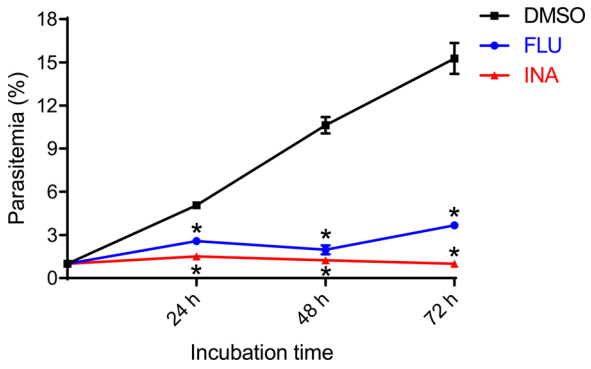

Fig. 2. Parasitemia of FLU- and INA-treated cells. Parasitemia was monitored using Giemsa-stained blood smears of the parasites treated with FLU and INA at concentrations of 2 × IC_{50}. DMSO (0.5%)-treated cells were used as the control group. The values are presented as mean ± S.D. of three independent experiments.

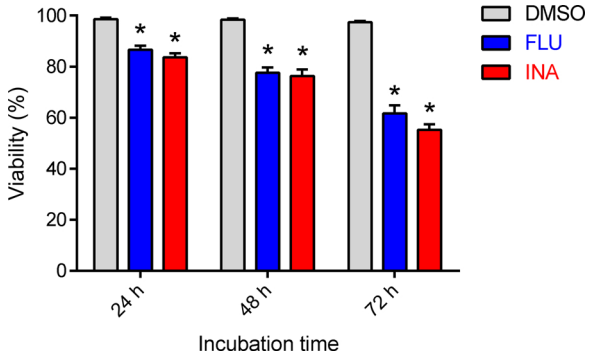

Fig. 3. Viability of FLU- and INA-treated cells. The viability of parasites treated with FLU and INA at concentrations of 2 × IC_{50} was determined by Hoechst 33,342/PI double staining. DMSO (0.5%)-treated cells were used as the control group. The values are presented as mean ± S.D. of three independent experiments.

IC_{50} to the parasite IC_{50}, of FLU was > 16.5, while that of INA was > 232.6, indicating that FLU and INA have low cytotoxicity for cells.

4. Discussion

The evolutionary relationship between apicomplexan parasites and plants is evidenced by many antimalarial drugs being herbicidal (Corral et al., 2017). Phytohormone inhibitors have also been reported to be drug candidates against apicomplexan parasites (Duvalsaint and Kyle, 2018).The initial report of a phytohormone inhibitor's activity on apicomplexan parasites described the activity of FLU against ABA of *T. gondii* (Nagamune et al., 2008a). Subsequently, the effects of FLU against *P. falciparum* and *N. caninum* were reported (Toyama et al., 2012; Ybañez et al., 2016). *P. falciparum* ABA, produced in blood stage parasites, plays a critical role in the regulation of Ca^{2+}-dependent release of both invasive merozoites and activated gametocytes (Glushakova et al., 2013). Thus, FLU reduces the levels of ABA and blocks the release of merozoites as well as of activated gametocytes (Billker et al., 2009). Importantly, another phytohormone inhibitor of GA synthesis, INA, also exerts strong inhibitory activities against both *T. gondii* and *P. falciparum* (Toyama et al., 2012).

The IC_{50} values of FLU and INA for *B. gibsoni* were 60.6 ± 3.4 and 4.3 ± 0.3 μM, respectively (Fig. 1). This result indicated that *B. gibsoni* was sensitive to FLU and INA, and that INA was more effective than FLU against *B. gibsoni*. Furthermore, both parasitemia and viability of FLU- and INA-treated cells were significantly lower ($^*p < 0.05$) than those of the control group at 24, 48, and 72 h after incubation (Figs. 2 and 3). The results showed that FLU and INA inhibited growth, leading to increased mortality of *B. gibsoni*. Moreover, the fluorescent micrographs obtained at 24 h after FLU and INA treatments showed that dead or dying parasites were stained by PI, compared to the DMSO-treated cells (Fig. 4). Dying parasites appeared white (merger of green, blue, and red) following the decrease of GFP level, before eventually appearing pink (merger of blue and red). However, there were no differences in morphological changes between the DMSO-treated cells and FLU- or INA-treated cells. In an additional experiment, the cytotoxicity of FLU

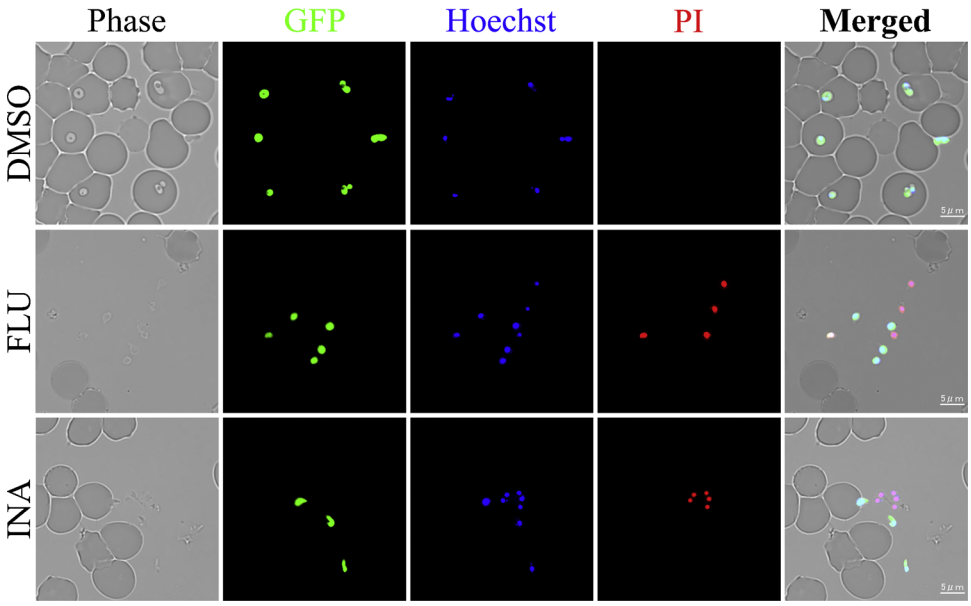

Fig. 4. Fluorescent microscope images of FLU- and INA-treated cells. The fluorescent micrographs of Bg-GFP were obtained at 24 h after FLU and INA treatments at concentrations of 2 × IC_{50}. Hoechst 33342 was used to stain the nuclei of both live and dead parasites. PI was used to stain the nuclei of dead parasites. DMSO (0.5%)-treated cells were used as the control group.

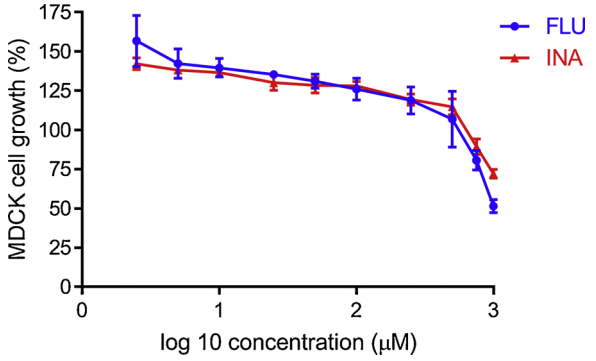

Fig. 5. Cytotoxicity assay of FLU and INA in the MDCK cell line. MDCK cells were exposed to FLU and INA at concentrations of 2.5–1000 μM. The medium (without cells) was used as the blank, and cells in the medium supplemented with 0.5% of DMSO was used as the control group. The values were preseted as mean ± S.D. of three independent experiments.

and INA was evaluated using the MDCK cell line. The IC_{50} values of both FLU and INA were higher than 1000 μM (Fig. 5). Therefore, FLU and INA showed low toxicity against the host cells and are thus safe.

Previous studies (Garg et al., 2014; He et al., 2018; Virji et al., 2018) have reported that *Babesia* parasites contain an apicoplast with a circular genome. Since there is no evidence for the presence of phytohormones, such as, ABA and GA in *B. gibsoni*, we were unable to reach a definitive conclusion on the target of FLU and INA in this parasite. However, because our results clearly indicated that FLU and INA inhibit the growth of *B. gibsoni* effectively, we believe that FLU and INA target phytohormones or similar signal molecules.

In summary, both FLU and INA showed effectiveness against *B. gibsoni in vitro*. Nonetheless, further studies are required to confirm the exact mechanism of action of these compounds against *B. gibsoni*.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

This study was supported by a Grant-in-Aid for Scientific Research (26304036 and 18H02336) and Japan Society for the Promotion of Science Core-to-Core program, both from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

References

Andrabi, S.B.A., Tahara, M., Matsubara, R., Toyama, T., Aonuma, H., Sakakibara, H., Suematsu, M., Tanabe, K., Nozaki, T., Nagamune, K., 2018. Plant hormone cytokinins control cell cycle progression and plastid replication in apicomplexan parasites. Parasitol. Int. 67, 47–58.
Arisue, N., Hashimoto, T., 2015. Phylogeny and evolution of apicoplasts and apicomplexan parasites. Parasitol. Int. 64, 254–259.
Baneth, G., 2018. Antiprotozoal treatment of canine babesiosis. Vet. Parasitol. 254, 58–63.
Billker, O., Lourido, S., Sibley, L.D., 2009. Calcium-dependent signaling and kinases in apicomplexan parasites. Cell Host Microbe 5, 612–622.
Corral, M.G., Leroux, J., Stubbs, K.A., Mylne, J.S., 2017. Herbicidal properties of antimalarial drugs. Sci. Rep. 7, 45871.
Duvalsaint, M., Kyle, D.E., 2018. Phytohormones, isoprenoids, and role of the apicoplast in recovery from dihydroartemisinin-induced dormancy of Plasmodium falciparum. Antimicrob. Agents Chemother. 62, e01771–17.
El-Dakhly, K.M., Goto, M., Noishiki, K., El-Nahass, el-S., Sakai, H., Yanai, T., Takashima, Y., 2015. Distribution patterns of *Babesia gibsoni* infection in hunting dogs from nine Japanese islands. J. Parasitol. 101, 160–166.
Garg, A., Stein, A., Zhao, W., Dwivedi, A., Frutos, R., Cornillot, E., Mamoun, C.B., 2014. Sequence and annotation of the apicoplast genome of the human pathogen Babesia microti. PLoS One 9, e107939.
Glushakova, S., Lizunov, V., Blank, P.S., Melikov, K., Humphrey, G., Zimmerberg, J., 2013. Cytoplasmic free Ca^{2+} is essential for multiple steps in malaria parasite egress from infected erythrocytes. Malar. J. 12, 41.
Goo, Y.K., Xuan, X., 2014. New Molecules in *Babesia gibsoni* and their application for diagnosis, vaccine development, and drug discovery. Korean J. Parasitol. 52, 345–353.
Gupta, R., Chakrabarty, S.K., 2013. Gibberellic acid in plant: still a mystery unresolved. Plant Signal. Behav. 8, e25504.
He, L., He, P., Luo, X., Li, M., Yu, L., Guo, J., Zhan, X., Zhu, G., Zhao, J., 2018. The MEP pathway in *Babesia orientalis* apicoplast, a potential target for anti-babesiosis drug development. Parasit. Vectors 11, 452.
Kjemtrup, A.M., Kocan, A.A., Whitworth, L., Meinkoth, J., Birkenheuer, A.J., Cummings, J., Boudreaux, M.K., Stockham, S.L., Irizarry-Rovira, A., Conrad, P.A., 2000. There are at least three genetically distinct small piroplasms from dogs. Int. J. Parasitol. 30, 1501–1505.
Kumagai, A., Zhang, G., Jia, H., Nakamura, C., Zhang, H., Goo, Y.K., Aboge, G.A., Terkawa, M.A., Zhou, J., Nishikawa, Y., Xuan, X., 2016. Expression of *Babesia gibsoni* thrombospondin-related adhesive protein in *Toxoplasma gondii* and evaluation of its antigenicity and immunogenicity. J. Protozool. Res. 26, 30–39.
Lim, L., McFadden, G.I., 2010. The evolution, metabolism and functions of the apicoplast. Philos. Trans. R. Soc. Lond. B Biol. Sci. 365, 749–763.
Liu, M., Adjou Moumouni, P.F., Asada, M., Hakimi, H., Masatani, T., Vudriko, P., Lee, S.H., Kawazu, S.I., Yamagishi, J., Xuan, X., 2018. Establishment of a stable transfection system for genetic manipulation of Babesia gibsoni. Parasit. Vectors 11, 260.
Matsubara, R., Aonuma, H., Kojima, M., Tahara, M., Andrabi, S.B., Sakakibara, H., Nagamune, K., 2015. Plant hormone salicylic acid produced by a malaria parasite controls host immunity and cerebral malaria outcome. PLoS One 10, e0140559.
McFadden, G.I., Yeh, E., 2017. The apicoplast: now you see it, now you don't. Int. J.

Parasitol. 47, 137–144.
Nagamune, K., Hicks, L.M., Fux, B., Brossier, F., Chini, E.N., Sibley, L.D., 2008a. Abscisic acid controls calcium-dependent egress and development in Toxoplasma gondii. Nature 451, 207–210.
Nagamune, K., Xiong, L., Chini, E., Sibley, L.D., 2008b. Plants, endosymbionts and parasites: Abscisic acid and calcium signaling. Commun. Integr. Biol. 1, 62–65.
Sato, S., 2011. The apicomplexan plastid and its evolution. Cell. Mol. Life Sci. 68, 1285–1296.
Shu, K., Zhou, W., Chen, F., Luo, X., Yang, W., 2018. Abscisic acid and gibberellins antagonistically mediate plant development and abiotic stress responses. Front. Plant Sci. 9, 416.
Solano-Gallego, L., Baneth, G., 2011. Babesiosis in dogs and cats–expanding parasitological and clinical spectra. Vet. Parasitol. 181, 48–60.
Sunaga, F., Namikawa, K., Kanno, Y., 2002. Continuous *in vitro* culture of erythrocytic stages of *Babesia gibsoni* and virulence of the cultivated parasite. J. Vet. Med. Sci. 64, 571–575.
Toyama, T., Tahara, M., Nagamune, K., Arimitsu, K., Hamashima, Y., Palacpac, N.M., Kawaide, H., Horii, T., Tanabe, K., 2012. Gibberellin biosynthetic inhibitors make human malaria parasite *Plasmodium falciparum* cells swell and rupture to death. PLoS One 7, e32246.
Virji, A.Z., Thekkiniath, J., Ma, W., Lawres, L., Knight, J., Swei, A., Roch, K.L., Mamoun, C.B., 2018. Insights into the evolution and drug susceptibility of *Babesia duncani* from the sequence of its mitochondrial and apicoplast genomes. Int. J. Parasitol.(Aug). https://doi.org/10.1016/j.ijpara.2018.05.008. pii: S0020-7519(18)30170-X [Epub ahead of print].
Ybañez, R.H., Leesombun, A., Nishimura, M., Matsubara, R., Kojima, M., Sakakibara, H., Nagamune, K., Nishikawa, Y., 2016. *In vitro* and *in vivo* effects of the phytohormone inhibitor fluridone against *Neospora caninum* infection. Parasitol. Int. 65, 319–322.