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MAJOR ARTICLE

An early treatment with BKI-1748 exhibits full protection against abortion and congenital infection in sheep experimentally infected with *Toxoplasma gondii*

Roberto Sánchez-Sánchez[a], Dennis Imhof[b], Yanina P. Hecker[a,c], Ignacio Ferre[a], Michela Re[a,d], Javier Moreno-Gonzalo[a,d], Javier Blanco-Murcia[a,d], Elena Mejías-López[d], Matthew A. Hulverson[e], Ryan Choi[e], Samuel L. M. Arnold[f], Kayode K. Ojo[e], Lynn K. Barrett[e], Andrew Hemphill[b], Wesley C. Van Voorhis[e], Luis Miguel Ortega-Mora[a]

[a]SALUVET, Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain.; [b]Institute of Parasitology, Vetsuisse Faculty, University of Berne, Länggass-Strasse 122, CH-3012 Berne, Switzerland.; [c]Institute of Innovation for Agricultural Production and Sustainable Development (IPADS Balcarce), INTA-CONICET, 7620, Balcarce, Argentina.; [d]Animal Medicine and Surgery Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain.; [e]Center for Emerging and Re-emerging Infectious Diseases (CERID), Division of Allergy and Infectious Diseases, Department of Medicine, University of Washington, Seattle, Washington, USA.; [f]Department of Pharmaceutics, University of Washington, Seattle, Washington, USA.

Congenital toxoplasmosis in humans and in some mammalian species, such as small ruminants, is a well-known cause of abortion and foetal malformations. The calcium-dependent protein kinase 1 (CDPK1) inhibitor BKI-1748 has shown a promising safety profile for its use in humans and a good efficacy against *Toxoplasma gondii* infection in vitro and in mouse models. The rates of congenital infection and foetal damage in sheep seem to mimic the situation in human

Correspondence: Luis Miguel Ortega Mora (luis.ortega@ucm.es), SALUVET, Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain. Phone number: +34 913944098.

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toxoplasmosis more closely than those in mouse models. Ten doses of BKI-1748 given every other day orally in sheep at 15 mg/kg exhibited therapeutic plasma levels for 23 days and no systemic or pregnancy-related toxicity was observed. In sheep experimentally infected at 90 days of pregnancy with a *T. gondii* oocyst dose which was lethal for all foetuses, the BKI-1748 treatment administered from 48 hours after infection led to complete protection against abortion and congenital infection. In addition, compared to infected/untreated sheep, treated sheep showed a drastically lower rectal temperature increase, higher IFNγ levels and none showed IgG seroconversion throughout the study. In conclusion, BKI-1748 treatment in pregnant sheep starting at 48 hours after infection was fully effective against congenital toxoplasmosis.

Keywords: *Toxoplasma gondii*; One Health therapeutics; BKI-1748; congenital toxoplasmosis; sheep; safety; efficacy

INTRODUCTION

Toxoplasmosis is a widespread zoonotic disease caused by the apicomplexan parasite *Toxoplasma gondii* [1]. Congenital *T. gondii* infections are a well-known cause of abortions and foetal malformations in humans and small ruminants [2, 3]. For the treatment of *T. gondii* infections in sheep, classical drugs were tested several decades ago [4]. These treatments were administered from ten days before challenge at ninety days of pregnancy (prophylactic use) until delivery and they showed low protection against abortion (20-40%) [5], and limited or no protection against congenital infection [5-8]. In humans, the collection of effective drugs (pyrimethamine, sulfadiazine, sulfadoxine, clindamycin, spiramycin) are of limited use as they cause significant adverse side effects and confer only moderate efficacy once infection is established. First-line combination treatment with pyrimethamine-sulfadiazine requires an extended treatment time and has been associated with allergic, hematologic, and nephrotoxic side effects, and teratogenicity (particularly in first-trimester pregnancy) [9-11]. These folate inhibitors may induce a folate deficiency, which is likely to be responsible for the side effects.

Calcium dependent protein kinase 1 (CDPK1) is present in apicomplexan parasites and it has homologs in plants and other phyla, but not in mammals [12-15]. CDPK1 activity is very essential in *T. gondii* host cell invasion and egress [12, 16, 17] and can be selectively targeted by a class of ATP-competitive compounds, collectively named bumped kinase inhibitors (BKIs). Apart from *T. gondii*, BKIs have activity against other important zoonotic apicomplexan parasites such *Cryptosporidium parvum* and *Plasmodium falciparum* since their CDPKs are structurally similar [18]. The pyrazolopyrimidine (PP) compound BKI-1294 showed high efficacy in vitro, in non-pregnant and pregnant mouse models of toxoplasmosis [19-22] and in a pregnant sheep model of toxoplasmosis [23]. However, BKI-1294 has some drawbacks, such as the inhibition of hERG (human ether-à-go-go related gene), that excludes its use in humans due to safety issues (cardiovascular risk) [24]. The compounds from the 5-aminopyrazole-4-carboxamide (AC) scaffold, such as BKI-1748, have shown lower affinity for hERG (acceptable

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cardiovascular safety testing in rat and dog, with only minor abnormalities detected above 18 μM) compared to BKIs with a PP scaffold, rendering this compound more safe and more promising [24, 25]. For the lead AC compound BKI-1748 the effective concentration inhibiting *T. gondii* tachyzoite proliferation in vitro by 50% (EC50) is in the range of 43-76 nM [25, 26]. Furthermore, BKI-1748 exhibited efficacy against *T. gondii* infection in mice [25] and in pregnant mouse models of toxoplasmosis and neosporosis [26].

Sheep models of congenital *T. gondii* infection have emerged as a good alternative to test the efficacy of drug candidates for the control of human toxoplasmosis since: i) the congenital infection/foetal damage rates following a primary infection in sheep [27] and probably humans [3] differ from that in mice [27, 28] and ii) the short period of gestation in mice and the large number of pups make the results difficult to extrapolate to humans [29, 30]. We report here on the safety and efficacy of BKI-1748 treatment in pregnant sheep experimentally infected with *T. gondii* oocysts at mid-gestation.

MATERIALS AND METHODS

Ethics statement

Protocols were approved by the Animal Welfare Committee (Community of Madrid, Spain, PROEX 064/19 and 210.0/22), following Spanish and European Union legislation (Law 32/2007, R.D. 53/2013, and Council Directive 2010/63/EU).

Experimental design

Experimental design was as previously described [23] with minor modifications (Table 1). Sheep were challenged at mid-pregnancy, 90 days of gestation (dg), with 1,000 *T. gondii* sporulated oocysts of the *T. gondii* isolate TgShSp1 (PCR-RFLP genotype 3) [27] and 48 hours later were treated with 10 doses of BKI-1748 (Fig. 1) at 15 mg/kg every 48 hours. Compound was dissolved at 30 mg/mL in a vehicle containing 60% PHOSAL® 53 MCT (medium-chain triglyceride emulsion), 30% PEG400 and 10% ethanol. In vivo (blood) and post-mortem samples were collected and for haematology and biochemistry we included an intermediate timepoint at 48 hours after the 5th dose of the compound. Safety of BKI-1748 was evaluated by monitoring of rectal temperatures, gastrointestinal (faecal consistency) and behavioural changes, foetal viability (including birthweights) and haematological and biochemical parameters. Efficacy of this compound was assessed by monitoring foetal mortality and congenital infection of the lambs. In addition, rectal temperatures and humoral and cellular immune responses to *T. gondii* infection were assessed.

Additionally, three pregnant sheep at 112-120 dg were used for catheterization of the saphenous vein of the foetus through surgery as previously described [31]. After recovery from the

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anaesthesia, the dams received an oral dose of 15 mg/kg of BKI-1748 in order to study the foetal pharmacokinetics (PK).

Study of PK, haematology/biochemistry, immune responses and parasite DNA detection

BKI-1748 plasma concentrations were determined as previously described [25]. Calculations of maximum concentration (C_{max}) for each dose, and area-under-the-curve (AUC) were determined using GraphPad Prism 8.0.1 software (San Diego, CA, USA).

As previously described [23], complete blood counts (CBCs) were determined in whole blood using the automated laser-based haematology analyser Advia 120 (Siemens, Healthcare Diagnostics GmbH, Eschborn, Germany). Biochemical parameters were measured in serum using the sequential automatic autoanalyzer Konelab 30 (Thermo Fisher Scientific, Waltham, USA). Ions were assessed in serum using a Microlyte 3 (Beckman Coulter, Brea, USA). Reference values were obtained from previous studies [32, 33].

Immune responses were assessed as previously described [23]. Briefly, IFNγ was evaluated by an enzyme-linked immunosorbent assay (ELISA), preceded by a peripheral blood stimulation assay using *T. gondii* soluble antigen, and *T. gondii*-specific IgG levels in dam and foetal sera were determined by ELISA and indirect fluorescent antibody test (IFAT), respectively. Genomic DNA extraction from 50–100 mg of six samples of different cotyledons (placenta) per dam and three samples of brain and lung tissues per foetus/lamb was carried out as previously described [23], using the commercial Maxwell® 16 Mouse Tail DNA Purification Kit (Promega, Wisconsin, USA). *T. gondii* DNA detection was carried out by an ITS1 PCR adapted to a single tube following procedures previously described [34].

Statistical analysis

Occurrence of foetal mortality was analysed by the Kaplan–Meier survival method and foetal survival curves were then compared by the Log-rank (Mantel-Cox) test. Rectal temperatures and cellular and humoral immunes responses were analysed using Two-way ANOVA of repeated measures test. Weights of the lambs were compared using the non-parametric Kruskal–Wallis test followed by Dunn's test for comparisons between groups, as well as the Mann–Whitney test for pairwise comparisons. AUC of PK curves were evaluated using the Mann–Whitney test. Haematological and biochemical parameters were compared between groups using One-way ANOVA test at each time point. Statistical significance for all analyses was established at $P < 0.05$. All statistical analyses were performed using GraphPad Prism 8.0.1 software (San Diego, CA, USA).

RESULTS

Pharmacokinetics

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BKI-1748 plasma concentrations in the dams are shown in Fig. 2A. A mean (± standard deviation) C_{max} of 2.7 ± 0.3 µM and 3.3 ± 0.3 µM were reached in the plasma of the dams at 8-24 hours (mainly at 12 hours) after each BKI-1748 administration in G1 (*T. gondii* infected and BKI-1748 treated) and G3 (uninfected and BKI-1748 treated group), respectively. Likewise, mean (± standard deviation) trough plasma concentrations of 0.7 ± 0.4 µM and 0.8 ± 0.2 µM at 48 hours after each BKI-1748 administration were found in G1 (*T. gondii* infected and BKI-1748 treated) and G3 (uninfected and BKI-1748 treated group), respectively. There was no significant difference between the AUC over the entirety of the dosing period for G1 (719.1 ± 301.9 h*µmol/L) and G3 (860.7 ± 322.0 h*µmol/L).

A comparison between maternal and foetal pharmacokinetics of BKI-1748 (Fig. 2B) showed that the C_{max} (at 20 hours after administration) was 1.8 ± 0.3 µM for pregnant sheep and 0.6 ± 0.01 µM for foetuses. The AUC through 20 hours after administration was 27.8 ± 0.2 h*µmol/L for pregnant sheep and 7.6 ± 1.2 h*µmol/L for foetuses, resulting in a ratio between dams and foetuses of 0.27 ± 0.04 h*µmol/L.

Haematology and biochemistry

Mean and standard deviations for haematological and biochemical parameters in G3 (uninfected/treated) and G4 (uninfected/vehicle alone) prior to treatment, during (48 hours after the 5th dose) and after treatment (4 days after the 10th dose) are shown in Table 2. Mean values for haematological and biochemical parameters were in the physiological range at initial, intermediate, and final time points, with no significant changes noted upon administration of BKI-1748.

Clinical observations

No changes in the rectal temperatures (Fig. 3), and no gastrointestinal or neurological clinical signs were observed in sheep that remained uninfected but received BKI-1748 treatment (G3) or the vehicle alone (G4). Likewise, no foetal mortality occurred in these two groups, and sheep gave birth to healthy lambs between 146 and 150 days of pregnancy. The birthweights of the lambs born from single pregnancies in G3 was 5450 ± 495 g. In twin pregnancies, birthweights of the lambs born were 3484 ± 277 g in G3 and 3898 ± 496 g in G4, with no significant differences between them. Finally, in the triplet pregnancy in G4, the weights of the newborn lambs were 1943 ± 361 g.

Concerning the infected groups, significant increase in rectal temperatures was observed between days 4 ($P < 0.001$) and 10 post infection (pi) ($P < 0.05$) in the *T. gondii*-infected, untreated G2 compared to the uninfected control G4. Comparing both infected groups, significantly lower rectal temperatures was observed in G1 (infected/treated) from day 5 ($P < 0.0001$) to 10 pi ($P < 0.05$) (Fig. 3).

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Foetal mortality was detected in 8 out of 8 sheep (100%) from infected but untreated group (G2), with 7 out of 8 sheep aborting on day 8 pi (early abortions) and the one remaining sheep suffered a chronic phase abortion on day 45 pi (three developed foetuses and one mummified foetus). However, in the infected and BKI-1748 treated group (G1), 7 out of 7 sheep (100%) gave birth to healthy lambs. Significant differences were found for foetal survival between G1 and G2 ($P < 0.0001$) (Fig. 4). The birthweights of the lambs born in G1 were 3767 ± 714 g and 3153 ± 533 g for single and twin pregnancies, respectively, with no significant differences compared to G3 and G4.

Cellular and humoral immune responses

IFNγ levels were significantly increased in culture supernatants from the infected/untreated group (G2) on days 10 ($P < 0.01$) and 14 pi ($P < 0.01$) compared to G4 (uninfected, vehicle-treated). When both infected groups were compared, significantly higher IFNγ levels were found on day 7 pi in G1 (infected/treated) compared to G2 (infected/untreated) ($P < 0.05$) (Fig. 5A).

Looking at the anti-*T. gondii* IgG levels in the dam´s sera (Fig. 5B), sheep from the infected/untreated group (G2) seroconverted on days 14 pi (2 out of 8 animals) or 21 pi (6 out of 8 animals). However, in G1 (infected/treated) none of the sheep (0/7) seroconverted throughout the study and remained at relative index percent (RIPC) values similar to the uninfected group G4. Comparing both infected groups, the infected and then treated sheep in G1 showed significantly lower IgG levels from the day 14 pi ($P < 0.001$) until the final sampling on day 56 pi ($P < 0.0001$). Foetuses from early abortions on day 8 pi in G2 (infected/untreated) and lambs born in G1 (infected/treated) were all seronegative by IFAT. However, the three aborted foetuses on day 45 pi in G2 (infected/untreated) were IgG seropositive with IFAT titres ranging from 1:8 to 1:32 (no thoracic liquid sample could be collected from the mummified foetus).

Parasite detection in placental and foetal tissues

In G2 (infected/untreated), parasite DNA was only detected in 1 out of 36 (2.8%) foetal brain samples from early abortions on day 8 pi. In addition, cotyledons or foetal lung samples tested from early abortions in G2 were PCR negative. By contrast, in the sheep suffering a chronic phase abortion on day 45 pi in G2, *T. gondii* DNA was detected in 4 out of 6 placental cotyledons (66.6%), and in all (9/9) foetal brain and foetal lung samples examined belonging to three foetuses. DNA samples from the mummified foetus were degraded and therefore PCR could not be carried out. In the infected/treated group G1, parasite DNA was neither detected in any of the samples from the placenta (cotyledons) nor the lambs (brain and lung tissues).

DISCUSSION

One Health integrative efforts should be practiced for an effective control of *T. gondii* infections in humans and animals [35, 36]. Preventing human exposure by reducing *T. gondii* in meat

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producing animals along with mitigating the risk of oocyst-acquired toxoplasmosis in humans, domestic animals, and wildlife are effective preventive measures [35]. For therapy, it is of great importance to identify novel, well-tolerated and efficacious drug candidates for the treatment of toxoplasmosis in both humans and animals.

BKI-1748 exhibits excellent pharmacokinetic properties. Based on the mouse PK and safety results [26], sheep were treated orally with a dosing regimen of 15 mg/kg every other day (q.o.d.), which resulted in a C_{max} of 2-3 µM and trough plasma concentrations of 0.7-0.8 µM. Furthermore, a successful drug candidate for the treatment of congenital toxoplasmosis requires both systemic maternal and foetal exposure. The foetal BKI-1748 plasma levels of 0.6 µM amounted up to around 30% of the plasma concentrations observed in the dams, similar to the amount of the compound previously described for BKI-1553 [31].

The application of BKI-1748 at a dosage of 15 mg/kg did not cause any adverse effects on pregnancy outcome in sheep. The resulting C_{max} in sheep (2-3 µM) was substantially lower than the values previously reported for in vitro toxicity [26, 37]. Zebrafish early life stages have emerged as a good alternative for toxicity testing [38]. However, for some BKIs, such as BKI-1748, detrimental effects on zebrafish embryo development do not correlate with pregnancy interference in mice maybe due to maternal health-related factors [37]. For BKI-1748, pregnancy interference in mice was observed after administration of 50 mg/kg/day for 5 days while treatments at 20 and 5 mg/kg/day were safe [26] and in the zebrafish embryo development model detrimental effects were noted at 20 µM and above [37, 39]. BKI-1748 did not inhibit hERG K+-channel activity at levels below 21.5 µM, thus there is no cardiovascular risk associated with treatment, and no inhibition at 10 µM was seen against SRC, a mammalian protein kinase with a small gatekeeper residue, suggesting little off-target kinase inhibition [25]. In dog cardiovascular testing, abnormalities such as positive inotropy, elevated heart rate and cardiac output as well as decreased systemic vascular resistance were found at levels above 18 µM [24].

Treatment in pregnant sheep was initiated at 2 days post-infection, in the same way as BKI-1748 therapy of *T. gondii* infection in non- pregnant [25] and pregnant mice [26] and as previously reported for BKI-1294 against congenital toxoplasmosis [23] and neosporosis [40] in sheep. However, contrary to these treatments in sheep in which 5 doses q.o.d. were administered, 10 doses of BKI-1748 were applied perorally q.o.d. The reasons for extending the treatment in vivo are based on i) the in vitro parasitostatic effect of BKIs [41] and ii) the fact that the lower number of doses using BKI-1294 resulted in only partial protection against congenital toxoplasmosis [23] and lack of protection against congenital neosporosis [40] due to reactivation of the infection after drug clearance.

Sheep that were infected at mid-pregnancy but non-treated exhibited 100% foetal mortality (87% early abortions and 13% classical/late abortions) as previously described [23, 27]. In contrast, in the infected and BKI-1748 treated group all sheep gave birth to healthy lambs that did not differ in the birthweights compared to uninfected ones. This full protection against foetal mortality

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after 23 days of BKI-1748 exposure marks an improvement compared to the previously reported 71% protection after 13 days of BKI-1294 exposure [23]. BKI-1748 also had shown high efficacy against pup mortality in CD1 mice (5.6% pup mortality in treated mice *vs* 40% pup mortality in untreated mice) [26]. However, CD1 mice appeared to be less susceptible in terms of offspring mortality than sheep [27].

Another clinical manifestation of *T. gondii* infection in sheep is the increase of rectal temperature early after primary infection with *T. gondii* oocysts. In the infected and BKI-1748 treated group lower rectal temperatures were observed from day 5 to day 10 pi compared to infected/untreated sheep, suggesting a great impact of BKI-1748 on parasite proliferation. However, in the previous BKI-1294 study [23], lower rectal temperatures were found only for a shorter time frame and with lower statistical significance than in the present study. In both BKI-efficacy studies we followed the same infection dose and day of treatment onset and similar plasma levels were found, and therefore it can be hypothesized that the better efficacy of BKI-1748 early after infection is related to an improved inhibition of parasite proliferation (*in vitro* EC50 of 43 nM for BKI-1748 and of 220 nM for BKI-1294) [21, 26]. The superior efficacy of BKI-1748 early after infection compared to BKI-1294 could also have led to the better outcomes in later stages of the infection.

In previous studies assessing the efficacy of BKIs against *Neospora caninum* [31, 40] and *T. gondii* [23] in sheep higher IFNγ production after in vitro stimulation of blood was found in infected and treated animals compared to infected but untreated sheep. Also, in the present study significantly higher IFNγ production on day 7 pi was found in blood cell culture supernatants of infected and treated sheep after in vitro stimulation with *T. gondii* antigens, although there was high individual variability. Concerning humoral immune responses, all infected but untreated sheep seroconverted on days 14-21 pi, similarly as previously described [27]. However, none of the infected and BKI-1748 treated sheep showed IgG seroconversion throughout the study. The lack of anti-*T. gondii* IgG after 23 days of BKI-1748 treatment contrasts with the IgG seroconversion of half of the animals after 13 days of BKI-1294 in the previous study [23], suggesting low or no reactivation of parasite proliferation after BKI-1748 clearance. In contrast BKI-1748 treatment in *T. gondii* infected pregnant mice did not prevent seroconversion [26].

The protection of BKI-1748 treatment against congenital *T. gondii* infection was also evaluated through foetal serology and parasite detection in placental and foetal tissues. In the infected but untreated animals, as previously described [27], foetuses suffering early abortions were seronegative and the parasites were rarely detected by PCR, while foetuses from chronic abortions were seropositive and PCR positive. The likelihood of TgShSp1 isolate to be transmitted congenitally in pregnant sheep is high since in a previous study all lambs born from sheep infected with only 10 oocysts were infected [27]. In the present study, the offspring from the infected and BKI-1748 treated group was seronegative and PCR negative, indicating that they were born non-infected. This is a clear improvement compared to the 46% infected lambs after BKI-1294 treatment [23] and also better protection than previously observed in the

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pregnant toxoplasmosis mouse model (5.6% positive pups in the treated group *vs* 63% in the untreated group) [26]. The lower protection rate of BKI-1748 in mice [26] compared to sheep in terms of offspring mortality and congenital infection, and the lack of seroconversion in sheep in contrast to mice, could be due to the more widespread systemic infection at the beginning of the treatment in mice. After oral infection with *T. gondii* oocysts, parasites were already detected in the mice placenta at 3 days pi [29], while in sheep there was no placental infection detected at day 7 after infection, partly because intestinal transit time is lower in mice [42] than in sheep [43].

The results showed that ten doses of BKI-1748 (15 mg/kg) every two days was safe in pregnancy and reached therapeutic systemic levels in the dams and the foetuses. For the efficacy, the treatment with the compound led to a full protection against congenital *T. gondii* infection since there was no transmission of the parasite to lambs and, in addition, treated infected sheep did not seroconvert. While BKI-1748 appears as a promising candidate for the treatment of toxoplasmosis, with satisfactory safety profile for its potential use in humans, follow-up studies in the experimental sheep model should define the minimal therapeutic exposure needed for a successful therapy. In addition, treatment regimens starting later after infection, after dissemination of the parasite in sheep has already taken place, should be evaluated.

NOTES

Author contributions: RSS, IF, AH, WVV and LMO conceived the study and participated in its design. RSS, MR and JMG selected the animals and executed the reproductive programme. RSS, DI, and YPH carried out oocyst infection and drug administration. RSS, DI, YPH, MR, JMG, JBM and EML participated in surgeries, clinical examination and in vivo sampling of the animals. RSS, DI and YPH performed necropsies and *post-mortem* collection of samples. MAH, RC, SA, KKO studied the plasma samples to determine the pharmacokinetics of the compound. RSS, DI and YPH performed the study of the immune response and the molecular detection of the parasite. RSS carried out the statistics and wrote the manuscript, with inputs in the discussion from IF, AH, LKB, WVV and LMO. All authors read and approved the final manuscript.

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Conflicts of interest: Dr. Wesley C. Van Voorhis is the President and co-owner of ParaTheraTech Inc., a company that is developing BKIs for animal health. Dr. Van Voorhis did not perform the experiments, nor interpret the results of the experiments, but he did edit this paper and helped plan the experiments. The other authors declare that they have no competing interests.

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REFERENCES

1. Dubey JP. Toxoplasmosis of animals and humans. 2nd ed. Boca Ratón FL: CRC Press, **2010**.
2. Dubey JP, Murata FHA, Cerqueira-Cezar CK, Kwok OCH, Su C. Economic and public health importance of *Toxoplasma gondii* infections in sheep: 2009-2020. Vet Parasitol **2020**; 286:109195.
3. Dubey JP, Murata F, Cerqueira-Cézar CK, Kwok O, Villena I. Congenital toxoplasmosis in humans: an update of worldwide rate of congenital infections. Parasitology **2021**; 148:1406-16.
4. Sanchez-Sanchez R, Vazquez P, Ferre I, Ortega-Mora LM. Treatment of toxoplasmosis and neosporosis in farm ruminants: state of knowledge and future trends. Curr Top Med Chem **2018**; 18:1304-1323.
5. Buxton D, Blewett DA, Trees AJ, McColgan C, Finlayson J. Further studies in the use of monensin in the control of experimental ovine toxoplasmosis. J Comp Pathol **1988**; 98:225-36.
6. Buxton D, Donald KM, Finlayson J. Monensin and the control of experimental ovine toxoplasmosis: a systemic effect. Vet Rec **1987**; 120:618-9.
7. Buxton D, Thomson KM, Maley S. Treatment of ovine toxoplasmosis with a combination of sulphamezathine and pyrimethamine. Vet Rec **1993**; 132:409-11.
8. Buxton D, Brebner J, Wright S, Maley SW, Thomson KM, Millard K. Decoquinate and the control of experimental ovine toxoplasmosis. Vet Rec **1996**; 138:434-6.
9. Konstantinovic N, Guegan H, Stäjner T, Belaz S, Robert-Gangneux F. Treatment of toxoplasmosis: Current options and future perspectives. Food Waterborne Parasitol **2019**; 15:e00036.
10. Shammaa AM, Powell TG, Benmerzouga I. Adverse outcomes associated with the treatment of *Toxoplasma* infections. Sci Rep **2021**; 11:1035.
11. Matok I, Gorodischer R, Koren G, Landau D, Wiznitzer A, Levy A. Exposure to folic acid antagonists during the first trimester of pregnancy and the risk of major malformations. Br J Clin Pharmacol **2009**; 68:956-62.
12. Lourido S, Shuman J, Zhang C, Shokat KM, Hui R, Sibley LD. Calcium-dependent protein kinase 1 is an essential regulator of exocytosis in *Toxoplasma*. Nature **2010**; 465:359-62.
13. Murphy RC, Ojo KK, Larson ET, et al. Discovery of Potent and Selective Inhibitors of CDPK1 from *C. parvum* and *T. gondii*. ACS Med Chem Lett **2010**; 1:331-5.
14. Ojo KK, Larson ET, Keyloun KR, et al. *Toxoplasma gondii* calcium-dependent protein kinase 1 is a target for selective kinase inhibitors. Nat Struct Mol Biol **2010**; 17:602-7.
15. Cardew EM, Verlinde CL, Pohl E. The calcium-dependent protein kinase 1 from *Toxoplasma gondii* as target for structure-based drug design. Parasitology **2018**; 145:210-8.

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16. Kieschnick H, Wakefield T, Narducci CA, Beckers C. *Toxoplasma gondii* attachment to host cells is regulated by a calmodulin-like domain protein kinase. J Biol Chem **2001**; 276:12369-77.
17. Lourido S, Tang K, Sibley LD. Distinct signalling pathways control *Toxoplasma* egress and host-cell invasion. EMBO J **2012**; 31:4524-34.
18. Van Voorhis WC, Doggett JS, Parsons M, et al. Extended-spectrum antiprotozoal bumped kinase inhibitors: A review. Exp Parasitol **2017**. 180:71-83.
19. Lourido S, Jeschke GR, Turk BE, Sibley LD. Exploiting the unique ATP-binding pocket of *Toxoplasma* calcium-dependent protein kinase 1 to identify its substrates. ACS Chem Biol **2013**; 8:1155-62.
20. Doggett JS, Ojo KK, Fan E, Maly DJ, Van Voorhis WC. Bumped kinase inhibitor 1294 treats established *Toxoplasma gondii* infection. Antimicrob Agents Chemother **2014**; 58:3547-9.
21. Winzer P, Muller J, Aguado-Martinez A, et al. *In Vitro* and *In Vivo* Effects of the Bumped Kinase Inhibitor 1294 in the Related Cyst-Forming Apicomplexans *Toxoplasma gondii* and *Neospora caninum*. Antimicrob Agents Chemother **2015**; 59:6361-74.
22. Müller J, Aguado-Martínez A, Ortega-Mora L, et al. Development of a murine vertical transmission model for *Toxoplasma gondii* oocyst infection and studies on the efficacy of bumped kinase inhibitor (BKI)-1294 and the naphthoquinone buparvaquone against congenital toxoplasmosis. J Antimicrob Chemother **2017**. 72:2334-41.
23. Sanchez-Sanchez R, Ferre I, Re M, et al. Treatment with Bumped Kinase Inhibitor 1294 Is Safe and Leads to Significant Protection against Abortion and Vertical Transmission in Sheep Experimentally Infected with *Toxoplasma gondii* during Pregnancy. Antimicrob Agents Chemother **2019**; 63:e02527-18.
24. Choi R, Hulverson MA, Huang W, et al. Bumped Kinase Inhibitors as therapy for apicomplexan parasitic diseases: lessons learned. Int J Parasitol **2020**. 50:413-422.
25. Hulverson MA, Bruzual I, McConnell EV, et al. Pharmacokinetics and *in vivo* efficacy of pyrazolopyrimidine, pyrrolopyrimidine, and 5-aminopyrazole-4-carboxamide bumped kinase inhibitors against toxoplasmosis. J Infect Dis **2019**; 219:1464-73.
26. Imhof D, Anghel N, Winzer P, et al. *In vitro* activity, safety and *in vivo* efficacy of the novel bumped kinase inhibitor BKI-1748 in non-pregnant and pregnant mice experimentally infected with *Neospora caninum* tachyzoites and *Toxoplasma gondii* oocysts. Int J Parasitol Drugs Drug Resist **2021**; 16:90-101.
27. Sánchez-Sánchez R, Ferre I, Regidor-Cerrillo J, et al. Virulence in mice of a *Toxoplasma gondii* type II isolate does not correlate with the outcome of experimental infection in pregnant sheep. Front Cell Infect Microbiol **2019**; 8:436.
28. Vargas-Villavicencio JA, Besné-Mérida A, Correa D. Vertical transmission and fetal damage in animal models of congenital toxoplasmosis: A systematic review. Vet Parasitol **2016**; 223:195-204.
29. Pfaff AW, Abou-Bacar A, Letscher-Bru V, et al. Cellular and molecular physiopathology of congenital toxoplasmosis: the dual role of IFN-γ. Parasitology **2007**; 134:1895-902.
30. Mukhopadhyay D, Arranz-Solís D, Saeij JP. Influence of the host and parasite strain on the immune response during *Toxoplasma* infection. Front Cell Infect Microbiol **2020**; 10:580425.
31. Sánchez-Sánchez R, Ferre I, Re M, et al. Safety and efficacy of the bumped kinase inhibitor BKI-1553 in pregnant sheep experimentally infected with *Neospora caninum* tachyzoites. Int J Parasitol Drugs Drug Resist **2018**; 8:112-24.

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32. Ramos-Antón JJ, Ferrer-Mayayo LM. La exploración clínica del ganado ovino y su entorno. Zaragoza: Servet, **2007**.
33. Smith BP, Van Metre D, Pusterla N. Large animal internal medicine 4th ed. St.Louis, MO: Elsevier **2009**: 406-412.
34. Hurtado A, Aduriz G, Moreno B, Barandika J, García-Pérez AL. Single tube nested PCR for the detection of *Toxoplasma gondii* in fetal tissues from naturally aborted ewes. Vet Parasitol **2001**; 102:17-27.
35. Aguirre AA, Longcore T, Barbieri M, et al. The One Health Approach to Toxoplasmosis: Epidemiology, Control, and Prevention Strategies. Ecohealth **2019**; 16:378-90.
36. de Barros RAM, Torrecilhas AC, Marciano MAM, Mazuz ML, Pereira-Chioccola VL, Fux B. Toxoplasmosis in human and animals around the world. Diagnosis and perspectives in the one health approach. Acta Trop **2022**; 231:106432.
37. Anghel N, Winzer PA, Imhof D, et al. Comparative assessment of the effects of bumped kinase inhibitors on early zebrafish embryo development and pregnancy in mice. Int J Antimicrob Agents **2020**; 56:106099.
38. Ducharme NA, Reif DM, Gustafsson J, Bondesson M. Comparison of toxicity values across zebrafish early life stages and mammalian studies: Implications for chemical testing. Reprod Toxicol **2015**; 55:3-10.
39. Müller J, Anghel N, Imhof D, et al. Common Molecular Targets of a Quinolone Based Bumped Kinase Inhibitor in *Neospora caninum* and *Danio rerio*. Int J Mol Sci **2022**; 23:2381.
40. Sánchez-Sánchez R, Ferre I, Re M, et al. A short-term treatment with BKI-1294 does not protect foetuses from sheep experimentally infected with *Neospora caninum* tachyzoites during pregnancy. Int J Parasitol Drugs Drug Resist **2021**; 17:176-85.
41. Müller J, Aguado-Martínez A, Balmer V, et al. Two novel calcium-dependent protein kinase 1 inhibitors interfere with vertical transmission in mice infected with *Neospora caninum* tachyzoites. Antimicrob Agents Chemother **2017**; 61:2324.
42. Padmanabhan P, Grosse J, Asad ABMA, Radda GK, Golay X. Gastrointestinal transit measurements in mice with 99mTc-DTPA-labeled activated charcoal using NanoSPECT-CT. EJNMMI Res **2013**; 3:1-8.
43. De Vega A, Gasa J, Castrillo C, Guada JA. Passage through the rumen and the large intestine of sheep estimated from faecal marker excretion curves and slaughter trials. Br J Nutr **1998**; 80:381-9.

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TABLE 1 Experimental design

Group	Number of pregnant sheep	Number of foetuses/lambs	Challenge (P.O.)	Treatment (P.O.)
G1	7	11	1000 TgShSp1 sporulated oocysts	BKI-1748, 10 doses at 15 mg/kg q.o.d., starting at 48 hours post-infection
G2	8	16	1000 TgShSp1 sporulated oocysts	None
G3	5	8	PBS	BKI-1748, 10 doses at 15 mg/kg q.o.d.
G4	3	7	PBS	60% Phosal 53 MCT®, 30% PEG400, 10% Ethanol 96ºC (vehicle), 10 doses q.o.d.

P.O.: *per os*, orally; q.o.d.: every other day

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TABLE 2 Haematological and biochemical parameters in sheep treated BKI-1748 and untreated

Parameter (units)	Reference values	G3 (uninfected/treated)			G4 (uninfected/vehicle alone)		
		Initial	Intermediate	Final	Initial	Intermediate	Final
Erythrocytes (x10^6)	9-14	10.75 ± 0.74	9.72 ± 1.01	9.78 ± 0.97	10.39 ± 1.68	9.87 ± 0.99	9.75 ± 1.03
MCH	8-12	10.70 ± 0.28	10.76 ± 0.65	11 ± 0.75	10.86 ± 0.45	10.70 ± 0.45	10.90 ± 0.62
MCHC	30-34	35.20 ± 1.55	34.98 ± 1.06	35.20 ± 1.07	34.16 ± 1.88	33.43 ± 2.02	33.40 ± 1.87
Haemoglobin (g/dL)	8-15	12.14 ± 1.32	10.86 ± 0.65	11.30 ± 0.62	11.23 ± 1.76	10.56 ± 1.40	10.81 ± 0.95
Packed cell volume (%)	28-40	34.42 ± 2.85	32.16 ± 3.23	32.80 ± 2.55	32.80 ± 3.38	32.40 ± 2.55	31.70 ± 2.03
Platelets (x10^3)	250-750	727.80 ± 205.20	637.80 ± 144.57	590.60 ± 92.96	489.33 ± 232.63	459.66 ± 136.69	377.66 ± 169.28
Leukocytes (x10^3)	4-12	5.96 ± 1.04	5.65 ± 0.64	6.04 ± 1.26	6.83 ± 0.38	7.29 ± 0.79	6.72 ± 0.57
Segment neutrophils (%)	10-50	38.68 ± 5.58	27.56 ± 4.12	34.92 ± 7.33	35.83 ± 6.42	34.03 ± 1.07	39.93 ± 8.31
Lymphocytes (%)	40-75	50.86 ± 8.20	59.16 ± 5.70	54.26 ± 6.45	53.20 ± 9.85	57.76 ± 1.41	52.13 ± 9.22
Monocytes (%)	1-6	5.72 ± 1.56	6.72 ± 3.81	7.22 ± 2.06	3.93 ± 2.87	3.03 ± 1.78	3.83 ± 1.05
Eosinophils (%)	0-15	2.52 ± 2.02	4.20 ± 1.76	2.28 ± 0.89	4.56 ± 1.53	2.56 ± 1.50	2.26 ± 0.72
Basophils (%)	0-3	0.30 ± 0.20	0.32 ± 0.08	0.20 ± 0.07	0.36 ± 0.05	0.30 ± 0.10	0.20 ± 0.10
Proteins (g/dL)	6-8	7.06 ± 0.36	6.58 ± 0.11	6.10 ± 0.35	7.30 ± 0.20	6.70 ± 0.52	6.06 ± 0.56
Albumin (g/dL)	3-4.5	3.34 ± 0.23	3.08 ± 0.17	2.98 ± 0.21	3.43 ± 0.11	3.10 ± 0.10	3.16 ± 0.11
AST (UI/L)	70-210	142.40 ± 37.38	100.60 ± 23.90	91.80 ± 25.26	93.33 ± 7.09	89.66 ± 18.92	74 ± 8.62
GGT (UI/L)	36-93	58.60 ± 10.87	55.20 ± 12.15	59 ± 12.44	53.33 ± 7.23	46.66 ± 4.50	47 ± 5.29
ALP (UI/L)	44-355	269.80 ± 92.21	255.60 ± 43.24	312.40 ± 104.70	209 ± 64.50	298.66 ± 137.53	266.33 ± 139.34
CK (UI/L)	50-180	159.40 ± 69.93	107 ± 26.30	75.80 ± 17.36	144.66 ± 107.77	85 ± 23.64	75.66 ± 10.11
Urea (mg/dL)	8.4-30.8	12.42 ± 3.46	6.58 ± 1.57	6 ± 1.68	12.03 ± 3.43	7.66 ± 1.20	13.53 ± 7.35
Creatinine (mg/dL)	0.9-1.7	1.24 ± 0.15	1.04 ± 0.05	0.96 ± 0.05	1.53 ± 0.41	1.40 ± 0.34	1.16 ± 0.46
Calcium (mg/dL)	7.1-9.8	9.98 ± 0.31	8.86 ± 0.96	9.82 ± 0.77	10.06 ± 0.60	9.73 ± 0.49	9.43 ± 0.15
Phosphorus (mg/dL)	3.5-7.3	6.58 ± 1.13	4.54 ± 0.65	6.22 ± 1.15	6.13 ± 1.96	4.76 ± 0.94	5.63 ± 1.09
Sodium (mEq/L)	139-152	148.20 ± 1.30	149 ± 3.53	150.60 ± 1.67	147.66 ± 1.15	148.66 ± 0.57	151.33 ± 1.15
Potassium (mEq/L)	3.9-5.2	5.26 ± 0.31	5 ± 0.18	5.14 ± 0.76	5 ± 0.45	4.70 ± 0.20	4.20 ± 0.43

Values are represented as Means ± S.D.

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FIGURES

FIG 1 Chemical structure of BKI-1748.

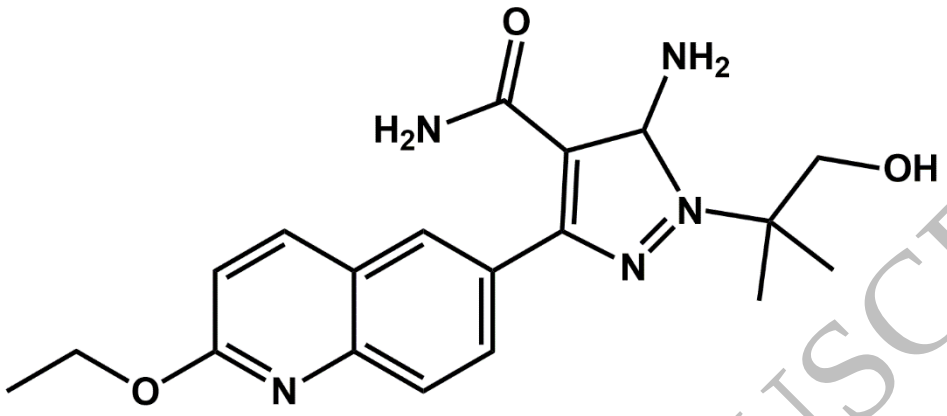

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FIG 2 BKI-1748 plasma concentrations in the infected/uninfected dams (A) and the foetuses (ratio dams/foetuses) (B). Mean + S.D. at the different sampling times are represented.

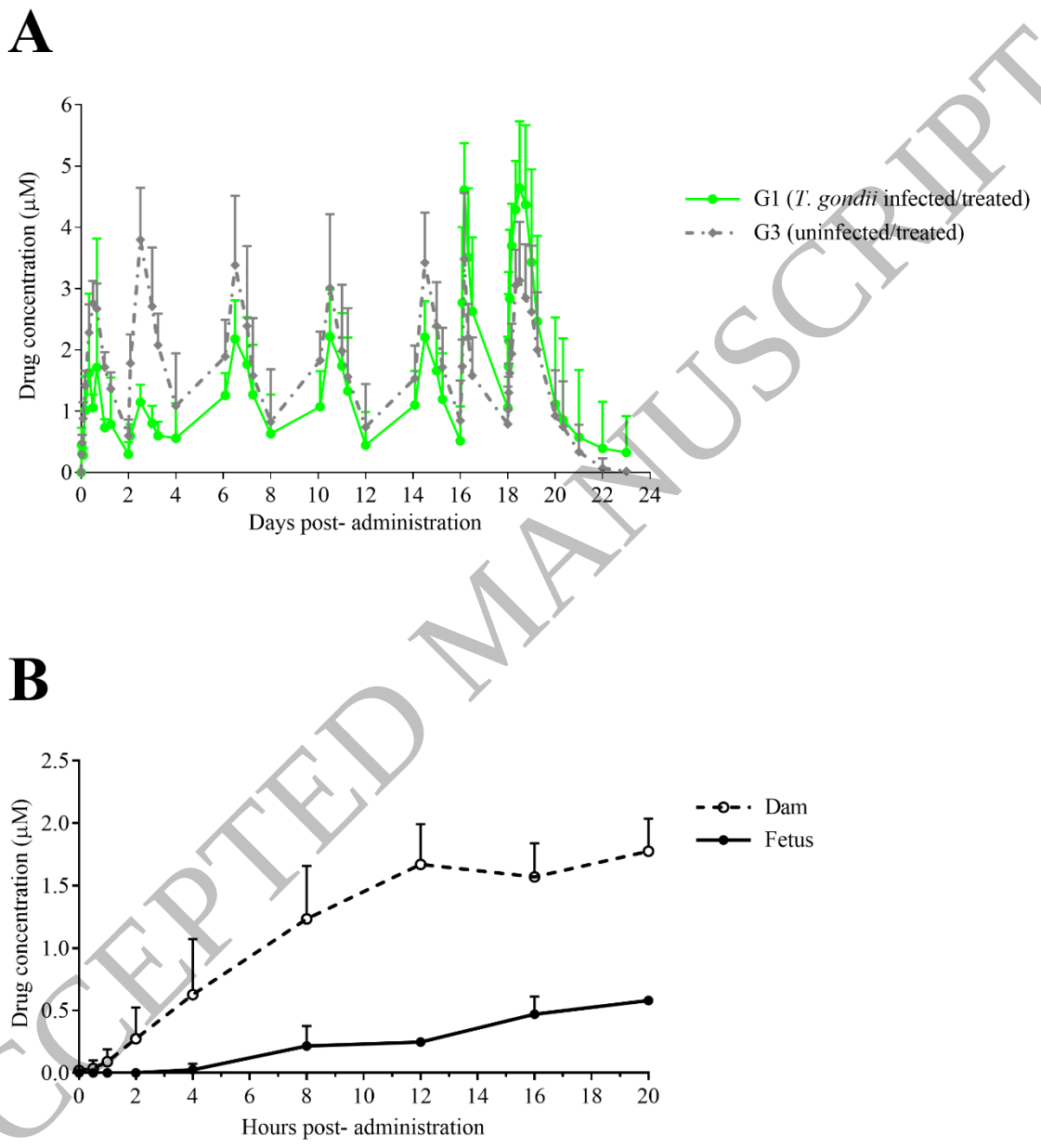

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FIG 3 Rectal temperatures of *T. gondii* infected and uninfected sheep, receiving or not receiving the treatment with BKI-1748. Each point represents the mean + S.D. for each group. Horizontal dashed line indicates the upper threshold for physiological rectal temperature in sheep. For significant differences, (*) indicates P < 0.05, (**) indicates P < 0.01 and (****) indicates P < 0.0001.

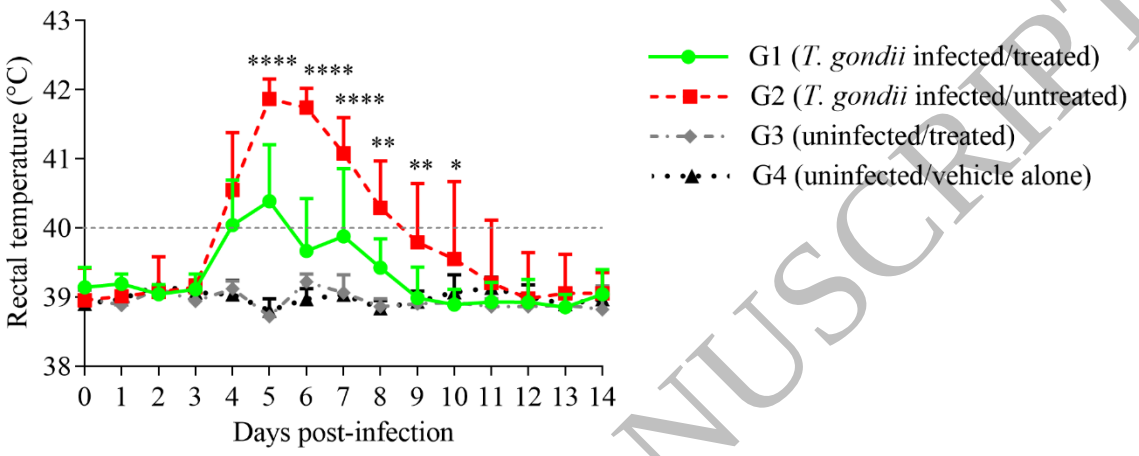

FIG 4 Kaplan–Meier foetal survival curves in *T. gondii* infected and uninfected sheep, receiving or not receiving the treatment with BKI-1748. Each point represents the percentage of surviving animals at that day, and downward steps correspond with observed deaths. For significant differences between foetal survival curves of infected groups, (****) indicates P < 0.0001.

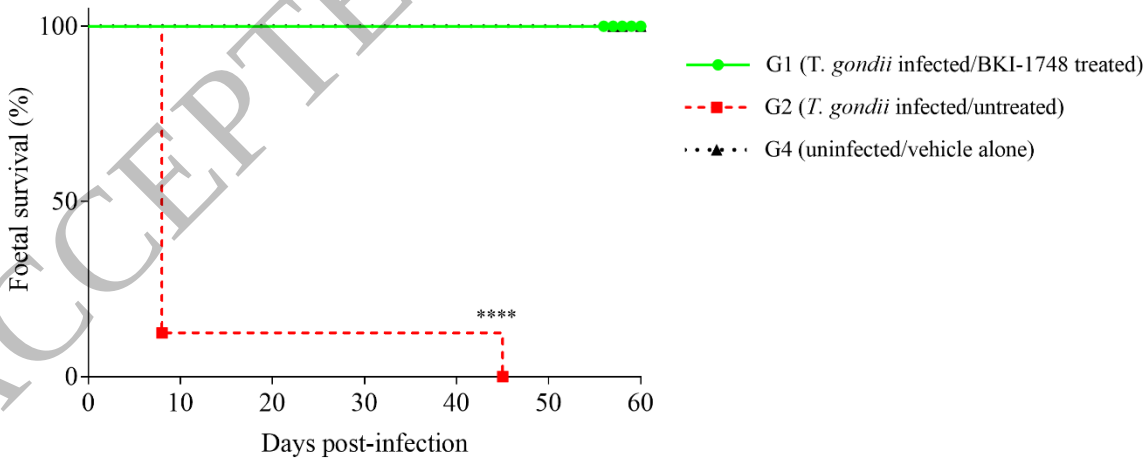

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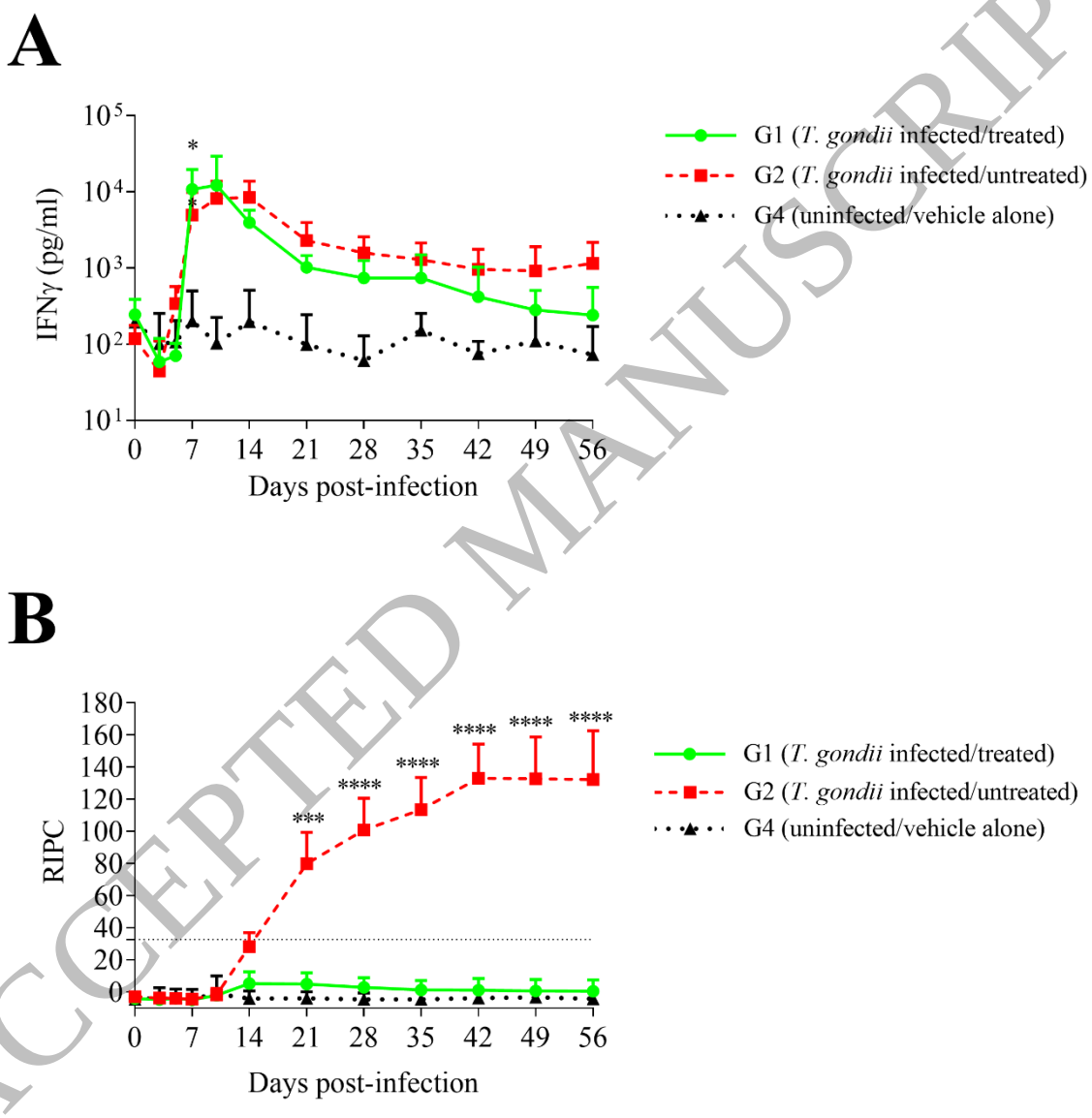
FIG 5 IFNγ in supernatants of peripheral blood cell cultures (A) and anti-*T. gondii* IgG levels in serum (B). In A, concentrations of IFNγ are expressed in pg/mL. In B, anti-*T. gondii* IgG levels are expressed in relative index percent (RIPC). Each point represents the mean + S.D. at the different sampling times for each group. Horizontal dashed line in B indicate the cut-off (RIPC ≥ 32.71). For significant differences between infected groups, (*) indicates P < 0.05, (***) indicates P < 0.001 and (****) indicates P < 0.0001.



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