A short-term treatment with BKI-1294 does not protect foetuses from sheep experimentally infected with *Neospora caninum* tachyzoites during pregnancy

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ABSTRACT

The *Neospora caninum* Calcium-dependent protein kinase 1 (NcCDPK1) inhibitor BKI-1294 had demonstrated excellent efficacy in a pregnant mouse model of neosporosis, and was also highly efficacious in a pregnant sheep model of toxoplasmosis. In this work, we present the efficacy of BKI-1294 treatment (dosed 5 times orally every 48 h) starting 48 h after intravenous infection of sheep with 10^5 Nc-Spain7 tachyzoites at mid-pregnancy. In the dams, BKI-1294 plasma concentrations were above the IC_{50} for *N. caninum* for 12–15 days. In treated sheep, when they were compared to untreated ones, we observed a minor increase in rectal temperature, higher IFNγ levels after blood stimulation *in vitro*, and a minor increase of IgG levels against *N. caninum* soluble antigens through day 28 post-infection. Additionally, the anti-NcSAG1 and anti-NcSAG4 IgGs were lower in treated dams on days 21 and 42 post-infection. However, BKI-1294 did not protect against abortion (87% foetal mortality in both infected groups, treated and untreated) and did not reduce transplacental transmission, parasite load or lesions in placentomes and foetal brain. The lack of foetal protection was likely caused by short systemic exposure in the dams and suboptimal foetal exposure to this parasitostatic drug, which was unable to reduce replication of the likely established *N. caninum* tachyzoites in the foetus at the moment of treatment. New BKIs with a very low plasma clearance and good ability to cross the blood-brain and placental barriers need to be developed.

1. Introduction

Neospora caninum infection is broadly recognized as one of the main infectious causes of abortion in cattle worldwide (Dubey and Schares, 2011; Dubey et al., 2017). In addition, according to recent information, *N. caninum* is also a relevant cause of reproductive failure in sheep flocks (West et al., 2006; González-Warleta et al., 2014). Transplacental crossing of the parasite after reactivation of a chronic infection during gestation is the main mode of transmission in cattle, although transmission through ingestion of oocysts (horizontal transmission) is also possible (Williams et al., 2009). Experimental infections in pregnant sheep (McAllister et al., 1996; Buxton et al., 1998; Weston et al., 2009; Arranz-Solís et al., 2015; Sánchez-Sánchez et al., 2018a) have shown that they are highly susceptible to *N. caninum* infection, with abortion and vertical transmission being the main consequences of infection.

To date, no vaccine or drugs are commercially licenced for the

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prevention of neosporosis. Triazinones and polyether ionophore antibiotics have been tested in nonpregnant ruminants experimentally infected with *N. caninum*, but the results were inconclusive (Kritzner et al., 2002; Haerdi et al., 2006; Vanleeuwen et al., 2011; Syed-Hussain et al., 2015). Novel treatment and vaccination options are drastically needed to reduce abortions caused by *N. caninum* and vertical transmission of the parasite (Horcajo et al., 2016; Sánchez-Sánchez et al., 2018b).

Novel approaches for the identification of drug targets in apicomplexan parasites have recently been developed (Müller and Hemphill, 2013). Calcium-dependent protein kinase 1 (CDPK1) activity is essential for microneme secretion, host cell invasion, and egress of *N. caninum* (Kieschnick et al., 2001; Lourido et al., 2010, 2012). CDPK1 is not found in mammals and therefore is an enticing target for drug development (Lourido et al., 2010; Murphy et al., 2010; Ojo et al., 2010; Cardew et al., 2018). Bumped kinase inhibitors (BKIs) are ATP-competitive compounds that selectively target CDPK1 in a broad spectrum of apicomplexan parasites (Van Voorhis et al., 2017, 2021; Choi et al., 2020). In sheep experimentally infected with *N. caninum* tachyzoites during pregnancy, BKI-1553 showed only a 37–50% reduction in abortion and a lack of protection against vertical transmission of the parasite. The *in vitro* efficacy for *N. caninum* was similar for the pyrazolopyrimidines BKI-1294 and BKI-1553, although they are not parasiticidal (Müller et al., 2017). However, BKI-1294 is a promising lead since it exhibited better efficacy in a pregnant mouse model of neosporosis (87% and 84% protection against pup mortality and vertical transmission, respectively) than BKI-1553 (20% and 13% protection against pup mortality and vertical transmission, respectively, using a safe dosage for pregnancy) (Winzer et al., 2015; Müller et al., 2017). Concerning side effects for pregnant sheep, subcutaneous administration of BKI-1553 showed transient dermal nodules (Sánchez-Sánchez et al., 2018c). In contrast, repeated oral administration of BKI-1294 to pregnant sheep was safe, both locally and systemically (Sánchez-Sánchez et al., 2019). In sheep experimentally infected with *Toxoplasma gondii* oocysts during pregnancy, BKI-1294 showed high efficacy against abortion and vertical transmission (71% and 53%, respectively) (Sánchez-Sánchez et al., 2019).

Recently, a refinement of the pregnant sheep model of neosporosis was carried out (Sánchez-Sánchez et al., 2018a). A tenfold reduction in the intravenous dose of Nc-Spain7 tachyzoites inoculated at mid-gestation compared to the dose used to test the efficacy of BKI-1553 in pregnant sheep (Sánchez-Sánchez et al., 2018c) also caused 100% abortion and vertical transmission (Sánchez-Sánchez et al., 2018a). Therefore, we report the efficacy of BKI-1294 treatment in sheep experimentally infected with a refined dose of Nc-Spain7 tachyzoites at mid-gestation.

2. Materials and methods

2.1. Ethics statement

This experiment involving animals was authorized by the Animal Welfare Committee of the Community of Madrid, Spain (PROEX 166/14), following procedures described in Spanish and European Union legal requirements (Law 32/2007, R.D. 53/2013, and Council Directive 2010/63/EU). Good clinical practices were implemented to guarantee animal welfare.

2.2. Animals and experimental design

Thirty pure Rasa Aragonesa breed female sheep aged 12 months were chosen from a commercial flock based on the seronegativity for *T. gondii*, *N. caninum*, Border disease virus (BDV), Schmallenberg virus (SBV), *Coxiella burnetii* and *Chlamydia abortus* by enzyme-linked immunosorbent assay (ELISA). They were oestrus-synchronized and mated with pure-breed Rasa Aragonesa tups for 2 days. Pregnancy and

foetal viability were confirmed by ultrasound scanning on day 40 post-mating, and twenty-one pregnant sheep were selected for the experiment. Animals were housed in the facilities of the Clinical Veterinary Hospital of Complutense University of Madrid (Spain). Pregnant ewes (n = 21) were randomly assigned to two experimentally infected groups (G1, n = 8; G2, n = 8) and an uninfected group (G3, n = 5). At 90 days gestation (dg), ewes from G1 and G2 were inoculated intravenously with 10^5 tachyzoites (passage 12) of the bovine Nc-Spain7 isolate (Sánchez-Sánchez et al., 2018a). Culture of Nc-Spain7 tachyzoites in MARC-145 cells and preparation of the inocula were carried out as previously described (Sánchez-Sánchez et al., 2018a). The remaining five uninfected pregnant sheep (G3) received an intravenous inoculum of phosphate-buffered saline (PBS) at 90 dg (Table 1).

BKI-1294 was synthesized by WuXi and further purified in the Department of Chemistry of the University of Washington (Seattle, USA). The drug was dissolved in a PHOSAL® 53 MCT (medium-chain triglyceride emulsion)-PEG400-ethanol vehicle as previously described (Sánchez-Sánchez et al., 2019). At 48 h post-infection (pi), BKI-1294 was administered orally 5 times every other day through an oro-ruminal probe to G1 (infected/treated) at a dose of 100 mg/kg bodyweight. In addition, sheep from G3 (uninfected/untreated) received 5 doses every other day of vehicle alone (Table 1).

2.3. Clinical monitoring and sample collection

During the trial, pregnant ewes were observed daily by a veterinarian. Rectal temperatures were measured daily from day 0 until 14 days pi and then weekly. Rectal temperatures over 40 °C were considered fever (Diffay et al., 2002). Safety parameters including gastrointestinal (faecal consistency and presence of blood in faeces) and behavioural changes were also examined.

BKI-1294 exposure was determined as previously described (Sánchez-Sánchez et al., 2019). Briefly, heparinized blood samples from G1 (infected/treated) were collected at different time points (Fig. 1), and drug plasma levels were analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS). To evaluate peripheral immune responses, blood samples were collected by jugular vein puncture into 5-mL vacutainer tubes (Becton Dickinson and Company, Plymouth, UK) with and without lithium heparin as an anticoagulant for the lymphoproliferation assay and to obtain serum samples, respectively. The time schedule for the sampling of G1 and G2 (both infected) and G3 (uninfected/untreated) was as follows: prior to infection, at 3, 5, 7 and 10 days pi and weekly afterwards.

Foetal viability (heartbeat and movements) pi was assessed by ultrasound scanning once a week. When foetal death occurred or 48 h after delivery, dams and lambs were sedated with xylazine (Rompun, Bayer, Mannhein, Germany) and then immediately euthanized by an intravenous overdose of embutramide and mebezonium iodide (T61, Intervet, Salamanca, Spain). Deliveries up to day 141 of pregnancy were considered premature. Live lambs were clinically monitored for 48 h after birth.

To assess vertical transmission of the parasite, brain pieces of

Table 1
Experimental design.

Group	Number of pregnant ewes	Challenge (I.V.)	Treatment (P.O.)
G1	8	10^5 Nc-Spain7 tachyzoites	BKI-1294, 5 doses at 100 mg/kg bodyweight every other day
G2	8	10^5 Nc-Spain7 tachyzoites	None
G3	5	PBS	60% Phosal 53 MCT®, 30% PEG400, 10% Ethanol (vehicle), 5 doses every other day

P.O.: *per os*.
MCT: medium-chain triglyceride emulsion.

foetuses/lambs in G1 (infected/treated), G2 (infected/untreated) and G3 (uninfected/untreated) were collected for DNA extraction and fixed for 5 days in 10% formalin for histopathological examination. In addition, six randomly selected placentomes were recovered from each placenta of aborted dams in G1 (infected/treated) and G2 (infected/untreated). For histopathological examinations, the placentomes were transversally cut into slices of 2–3 mm thickness and stored for 5 days in 10% formalin. The rest of the placentomes were stored for parasite DNA detection by PCR. In the dams that gave birth in G1 (infected/treated), G2 (infected/untreated) and G3 (uninfected/untreated), six randomly selected cotyledons were recovered for parasite DNA detection. Additionally, the IgG levels in the foetuses/lambs were used to determine the vertical transmission of the parasite. For this, thoracic fluids were collected from aborted foetuses in G1 (infected/treated) and G2 (infected/untreated), and precolostral serum was collected from lambs born in G1 (infected/treated), G2 (infected/untreated) and G3 (uninfected/untreated). Samples collected for evaluation of drug exposure, parasite DNA detection in tissues and anti-*N. caninum* IgG levels in the dams and the foetuses were maintained at −80 °C until analysis.

2.4. Evaluation of BKI-1294 plasma protein binding

Plasma protein binding (PPB) was determined as previously described (Tatipaka et al., 2014) with the following modifications. Test compound in DMSO was added to 120 μL of plasma to achieve a concentration of 5 μM. 20 μL of plasma spiked with compound was taken as a 100% recovery standard. The remaining 100 μL of spiked plasma was added to one side (donor) of the dialysis membrane sheet (MW cutoff 3.5 kDa, HTDialysis catalog no. 1135). 100 μL of dialysis buffer (14.2 g/L Na_2HPO_4, 8,77 g/L NaCl, pH 7.4) was added to the other side (acceptor) of the membrane sheet. After incubation, 20 μL aliquots were taken from the donor side and 80 μL aliquots were taken from the acceptor side. Samples were matrix adjusted by adding either blank plasma or blank buffer. BKI-1294 was extracted using 80% acetonitrile-20% water with internal standard and centrifuged to precipitate protein and extract compound. Supernatant was re-plated and compound measured with an Acquity ultra-performance liquid chromatography (UPLC) system in tandem with a Xevo TQ-S micro mass spectrometer (Waters, Milford, MA, USA). Fraction of compound bound to protein was calculated as bound/(unbound + bound) while adjusting for the matrix adjustment dilution.

2.5. BKI-1294 pharmacokinetics

Plasma samples from G1 (infected/treated) were processed, and BKI-1294 levels were measured with an Acquity ultra-performance liquid chromatography (UPLC) system in tandem with a Xevo TQ-S micro mass spectrometer (Waters, Milford, MA, USA) as previously described (Sánchez-Sánchez et al., 2019).

2.6. Peripheral blood cell stimulation assay and assessment of interferon-gamma (IFNγ) production

Peripheral blood stimulation assays and assessments of IFNγ production were carried out as previously described (Sánchez-Sánchez et al., 2018c). Briefly, heparinized blood was cultured in the presence of either soluble *N. caninum* antigens or concanavalin A (ConA, Sigma-Aldrich, Madrid, Spain). After incubation in a 5% CO_2/37 °C/100% humidity atmosphere for 24 h, recovered supernatants were tested for IFN-γ detection using a commercial enzyme immunoassay (Mabtech AB, Sweden).

2.7. Serological evaluation

In the dams, IgG levels against *N. caninum* soluble antigens were assessed using an in-house indirect ELISA, as previously described (Sánchez-Sánchez et al., 2018c), but with slight modifications. In summary, soluble *N. caninum* antigens were used to coat 96-well microtitre plates (Thermo Fisher Scientific, Waltham, USA). Plates were blocked, and serum samples were diluted 1:100 using 1% bovine serum albumin diluted in PBS containing 0.05% Tween 20 (PBS-T). Then, horseradish peroxidase-conjugated monoclonal anti-goat/sheep IgG (A9452-Sigma-Aldrich, Madrid, Spain) diluted 1:20000 in PBS-T was added, and later, ABTS (Roche, Basilea, Switzerland) was used as the substrate. The reaction was stopped by 0.3 M oxalic acid, and the optical density (OD) was read at 405 nm (OD405). OD values were converted into a relative index percent (RIPC) using the formula: RIPC = (OD405 sample-—OD405 negative control)/(OD405 positive control– OD405 negative control) × 100.

On days 0, 21, and 42 pi, anti-NcSAG1 and anti-NcSAG4 IgGs were assessed in the dams by ELISA and WB (Western blot), respectively. We used the commercial ELISA "ID Screen® toxoplasmosis indirect multi-species" (IDvet, Grabels, France) coated with TgSAG1. Commercial ELISA was carried out following the manufacturer's guidelines, and OD values were converted into S/P percentages using the following formula: S/P % = (OD450 sample – OD450 negative control)/(OD450 positive control – OD450 negative control) x 100. For *T. gondii*, an S/P % ≥ 50 indicates a positive result. As NcSAG1 is homologous to TgSAG1 (Howe et al., 1998; Dong et al., 2013) and a recent study already described cross reaction between TgSAG1 and anti-*N. caninum* antibodies using sheep sera (Huertas-López et al., 2021) we named anti-NcSAG1 antibodies to those ones that recognized TgSAG1 protein.

The presence of IgG against the bradyzoite antigen NcSAG4 was assessed by WB. SDS-PAGE was performed similarly to Aguado-Martínez et al. (2008) but under reducing conditions using dithiothreitol (DTT). Five micrograms of NcSAG4 recombinant protein expressed in *Escherichia coli* (Novagen, Darmstadt, Germany) was electrophoresed in 15% acrylamide/bis-acrylamide minigels and transferred to nitrocellulose membranes (Mini Trans-Blot cell; Bio-Rad Laboratories, CA, USA). Nitrocellulose membranes were cut in strips. WB was carried out similarly to the method described for *N. caninum* (Álvarez-García et al., 2002). Serum samples were used at a 1:20 dilution, and horseradish peroxidase-conjugated monoclonal anti-goat/-sheep IgG (A9452-Sigma-Aldrich, Madrid, Spain) was diluted 1:8000.

In foetuses and lambs, anti-*N. caninum* IgG levels were measured in foetal fluids and precolostral sera, respectively, using an indirect fluorescent antibody test (IFAT) described by Álvarez-García et al. (2003) and used in previous studies (Sánchez-Sánchez et al., 2018a, 2018c).

2.8. Histopathology

Placentomes and foetal brains were processed for histological evaluation using haematoxylin and eosin (HE) staining. In addition, characteristic lesions found in both tissue samples from G1 (infected/treated) and G2 (infected/untreated) were measured using ImageJ software as previously described (Arranz-Solís et al., 2015). The number of foci (foci/cm^2), the average size of the lesions (ASL) and the total area of the lesion (%LES) were calculated for each tissue.

2.9. DNA extraction and PCR for parasite detection and quantification

Genomic DNA was extracted from 50 to 100 mg of foetal brain (three replicates of each) and placental samples (six samples of the placentomes in aborted dams or cotyledons in dams that gave birth) using

the sequential capture and release of Magnesil® Paramagnetic Particles available in the commercial Maxwell® 16 Mouse Tail DNA Purification Kit, developed for the automated Maxwell® 16 System (Promega, WI, USA). The DNA concentration of each sample was determined using a Synergy® H1 multimode microplate reader (Biotek, Winooski, USA) and Gen5 version 2.09.1 software (Biotek, Winooski, USA) and adjusted to 100 ng/μL in RNase-, DNase-, and protease-free water. *Neospora caninum* DNA detection was carried out in 5 μL of sample DNA by PCR amplification of the Nc5 region (Müller et al., 1996) using Np21plus- and Np6plus-specific primers and a final volume of 25 μL. Positive PCR controls with *N. caninum* genomic DNA equivalent to 10 (n = 1), 1 (n = 3) and 0.1 (n = 2) tachyzoites in 100 ng of sheep genomic DNA were included in each PCR. As negative controls, one sample from G3 (uninfected/untreated) was included in each round of DNA extraction and PCR. Ten-to 15-μL aliquots of the Nc5-PCR products were visualized under UV light in a 1.5% agarose gel stained with GelRed® nucleic acid gel stain (Biotium Inc., Fremont, USA) to detect the *N. caninum*-specific 337 bp amplification product. Validation of each nested Nc5-PCR assay required the absence of amplification of the sample from the uninfected group and the amplification of at least two positive controls (10 and 1 tachyzoites).

Positive placental and foetal brain samples by Nc5-PCR were quantified using real-time PCR (qPCR) in the ABI 7500 Fast Real-time PCR System (Applied Biosystems, Foster City, CA, USA). Primer pairs for parasite and host DNA quantification had been previously described (Collantes-Fernández et al., 2002; Gutierrez et al., 2012). Amplification reactions were performed as described Sánchez-Sánchez et al. (2018c), and parasite burden was expressed as parasite number/mg ovine tissue.

2.10. Statistical analysis

Rectal temperatures were compared using two-way repeated-measures ANOVA until 14 days pi and one-way ANOVA afterwards. Differences in IFNγ and IgG levels against *N. caninum* soluble antigens in the dams were tested using two-way ANOVA of repeated measures testing until 28 days pi and one-way ANOVA afterwards. Comparison of IgG levels against NcSAG1 was carried out by one-way ANOVA followed by Tukey's multiple comparisons test. The median foetal survival time (day at which 50% of the foetuses aborted) was calculated for the infected groups (G1 and G2). Foetal mortality was analysed by the Kaplan–Meier survival method, and foetal survival curves were compared by the log-rank (Mantel-Cox) test. Differences in the frequency of PCR detection of parasite DNA by Nc5-PCR and the number of animals with specific lesions were evaluated using the $\chi 2$ or Fisher's exact F-test. Differences in the humoral immune responses in the foetuses/lambs (inverse value of the IFAT titres), parasite burdens and histological measurements of lesions in infected groups (G1 and G2) were analysed using the Mann–Whitney test. Statistical significance for all analyses was established at $P < 0.05$. All statistical analyses were performed using GraphPad Prism 6.01 software (San Diego, CA, USA).

3. Results

3.1. Pharmacokinetics

In G1 (infected/treated), maximum concentrations (C_{max}) of 2.1 ± 0.7 μM, 2.1 ± 0.7 μM, 2.4 ± 1.2 μM, 2.7 ± 0.9 μM, and 3.1 ± 1 μM were reached at 8–30 h after the 1st, 2nd, 3rd, 4th, and 5th doses, respectively. Furthermore, trough plasma concentrations of 0.7 ± 0.4 μM, 0.7 ± 0.4 μM, 0.9 ± 0.5 μM, 1.4 ± 1.1 μM, and 1.3 ± 0.8 μM 48 h after the 1st, 2nd, 3rd, 4th, and 5th doses, respectively, and measurable drug levels until 6–7 days after the last dose were found (Fig. 1). There was a period of 12–15 days in which the plasma concentrations were above the half-maximal inhibitory concentration (IC_{50}).
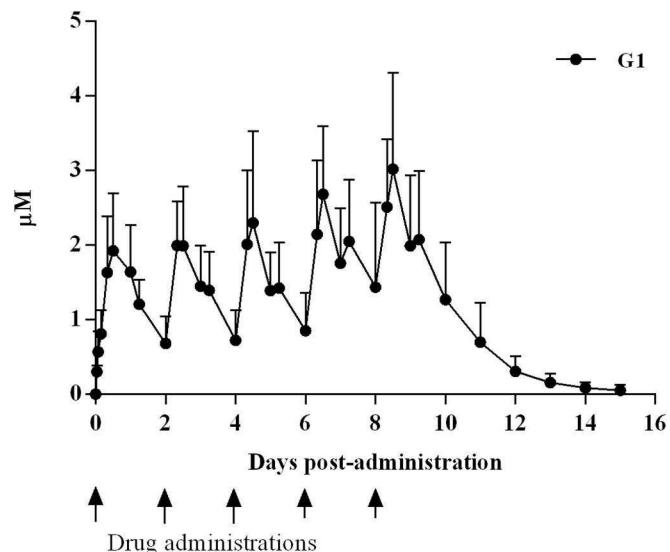

Fig. 1. BKI-1294 plasma exposure in *N. caninum*-infected and treated sheep. Each point represents the mean + S.D.

3.2. Plasma protein binding of BKI-1294

The PPB often affects the compound clearance, as more tightly bound compounds are less available for hepatic and renal clearance. In addition, PPB is sometimes hypothesized to influence the efficacy of antimicrobial compounds, as the free, unbound fraction is more readily available to engage with drug targets in the microbe. We tested the PPB of BKI-1294 against sheep, mouse, rat, dog and human plasma. Dog plasma bound less BKI-1294 (52% bound) than sheep (78% bound), and sheep plasma bound less BKI-1294 than mouse (85% bound), rat (85%) or human (85%) plasma.

3.3. Rectal temperatures and safety monitoring

Significantly increased rectal temperatures were found between days 5 ($P < 0.001$) and 8 pi ($P < 0.001$) in both infected groups compared to the uninfected control group G3. Comparing infected groups, on days 5, 6, 7, and 8 pi in G2 (infected/untreated), 6–7 out of 8 sheep showed fever, while in G1 (infected/treated), fever was found only in 3–5 out of
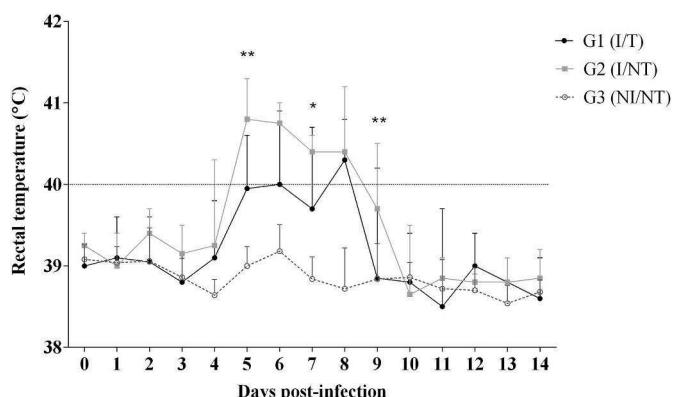

Fig. 2. Rectal temperatures of *N. caninum*-infected (G1 and G2) and uninfected sheep (G3). In the infected groups, G1 (I/T) received BKI-1294 treatment, and G2 (I/NT) did not. The uninfected group (G3) received vehicle alone (NI/NT). Each point represents the mean ± S.D. for each group. Represented rectal temperatures were analysed using two-way repeated-measures ANOVA. For significant differences, (*) indicates $P < 0.05$ and (**) indicates $P < 0.01$.

8 sheep on these days. A significant minor increase in rectal temperature was observed in G1 (infected/treated) on days 5 ($P < 0.01$) and 7 ($P < 0.05$) and 9 pi ($P < 0.01$) compared to G2 (infected/untreated) (Fig. 2). From day 14 pi until the end of the experiment, no changes in rectal temperatures were found. Diarrhoea, bloody stools, and behavioural disorders were not observed in any sheep.

3.4. Cellular and humoral immune responses in the dams

In the analysed supernatants from blood cell cultures stimulated with *N. caninum* antigen, IFNγ was significantly increased on day 7 pi in both infected groups ($P < 0.01$) compared to the uninfected and untreated group (G3), which showed baseline levels during the study. When infected groups were compared, G1 (infected/treated) exhibited higher IFNγ levels on day 7 pi than G2 (infected/untreated) ($P < 0.0001$). Although the comparison on days 14, 21 and 28 pi did not reveal significant differences, the IFNγ levels in G1 (infected/treated) were


A

B

C
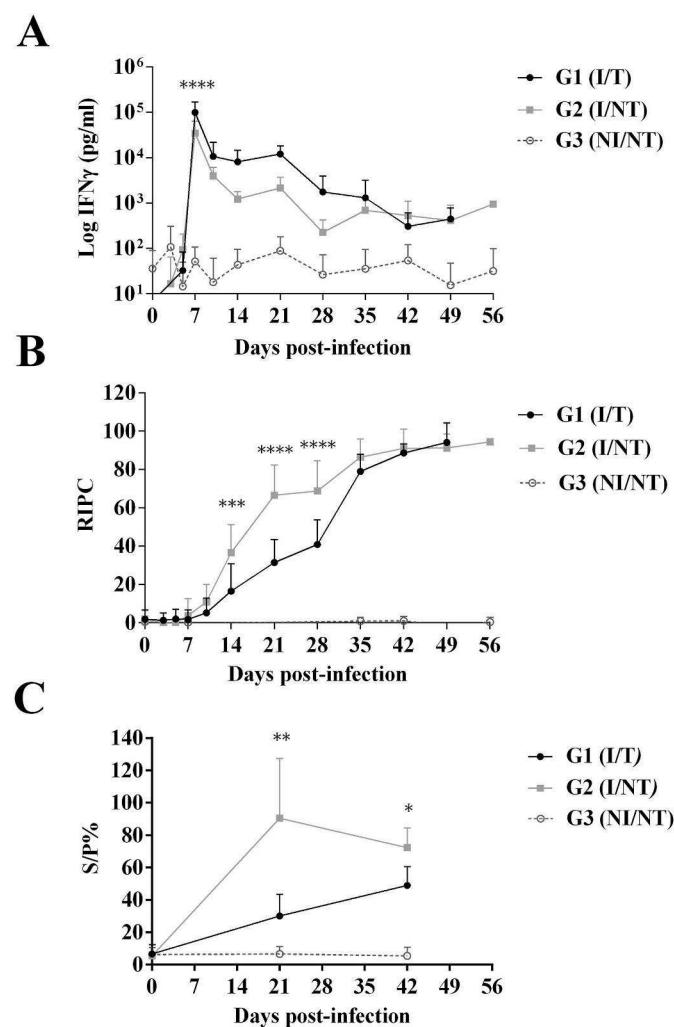
Fig. 3. *N. caninum*-specific cellular and humoral immune responses in the dams. ELISA values for IFNγ in supernatants of peripheral blood cell cultures, IgG levels in sera against *N. caninum* soluble antigens and anti-NcSAG1 antibodies in sera are represented in A, B and C graphs, respectively. Two of the eight dams in G1 (infected/treated) and G2 (infected/untreated) could not be tested for anti-NcSAG1 antibodies since there was not enough serum. Each point represents the mean ± S.D. at the different sampling times for each group. For significant differences between infected groups, (*) indicates $P < 0.05$, (**) indicates $P < 0.01$, (***) indicates $P < 0.001$ and (****) indicates $P < 0.0001$. I/T means infected/treated, I/NT means infected/non-treated and NI/NT means non-infected/non-treated.

approximately 10-fold higher on these days than in G2 (infected/untreated) (Fig. 3A).

Concerning the humoral immune response in the dams, IgG levels against *N. caninum* soluble antigens rose significantly from day 14 pi onwards in G1 (infected/treated) ($P < 0.001$) and G2 (infected/untreated) ($P < 0.0001$) compared to the uninfected and untreated animals (G3). Comparison of IgG levels against *N. caninum* soluble antigens in infected animals revealed a lower antibody response in G1 (infected/treated) than in G2 (infected/untreated) on days 14 ($P < 0.001$), 21 ($P < 0.0001$) and 28 pi ($P < 0.0001$). From day 35 pi onwards, no significant differences in anti-*N. caninum* IgG levels against *N. caninum* soluble antigens were found between both infected groups (Fig. 3B).

Anti-NcSAG1 antibodies were found in the *N. caninum*-infected animals using the commercial ELISA "ID Screen® toxoplasmosis indirect multispecies". All analysed serum samples from dams in G2 (*N. caninum* experimentally infected/untreated) were positive on day 21 and/or 42 pi according to the cut-off of the commercial kit for *T. gondii* (S/P % ≥50). All serum samples from G1 (infected/treated) and G2 (infected/untreated) on day 0 pi and from G3 (uninfected/untreated) on days 0, 21 and 42 pi were negative. Comparing anti-NcSAG1 IgG levels between experimentally infected animals, lower anti-NcSAG1 IgG levels were found in the dams from G1 (infected/treated) compared to G2 (infected/untreated) on days 21 ($P < 0.01$) and 42 pi ($P < 0.05$) (Fig. 3C).

Regarding anti-NcSAG4 IgGs detected by WB, all analysed serum samples from dams in G2 (infected/untreated) were moderately to highly positive on days 21 and 42 pi. However, lower recognition of NcSAG4 was found in the serum samples from dams in G1 (infected/treated), with 4 out of 6 serum samples weakly positive on day 21 pi and 3 out of 5 serum samples weakly to moderately positive on day 42 pi. No recognition of NcSAG4 was found in the serum samples from day 0 pi tested (Supplementary file 1).

3.5. Foetal survival

In G1 (infected/treated), as well as in G2 (infected/untreated), foetuses from 7 out of 8 sheep (87%) died. Foetal death was detected between 29 and 52 days pi in G1 (infected/treated) (median day 44 pi) and between 28 and 52 days pi in G2 (infected/untreated) (median day 45 pi). Therefore, no significant differences were found in the foetal survival rate associated with BKI-1294 administration. Healthy lambs were born from one sheep in G1 (infected/treated) (on day 141 of pregnancy, premature) and from one sheep in G2 (infected/untreated) (on day 151 of pregnancy). Dams from the uninfected group (G3) gave birth to healthy offspring between days 146 and 150 of pregnancy, and its foetal survival rate was significantly higher than the foetal survival rate of both
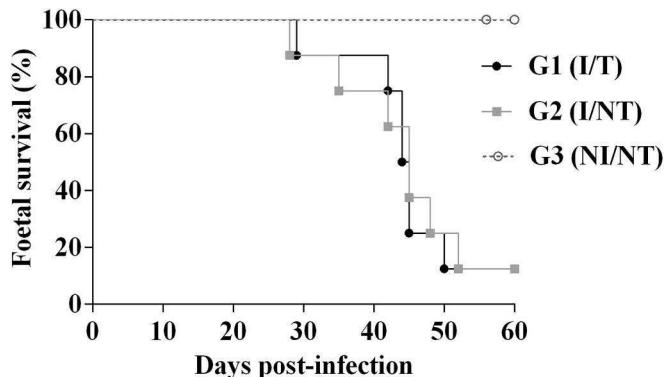

Fig. 4. Kaplan–Meier survival curves for foetuses in the *N. caninum*-infected (G1 and G2) and uninfected groups (G3). G1 (I/T) received BKI-1294 treatment, and G2 (I/NT) did not. The uninfected group (G3) received vehicle alone (NI/NT). Each point represents the percentage of surviving foetuses on that day, and downward steps correspond to observed foetal deaths. Foetal survival curves were compared by the log-rank (Mantel-Cox) test.

infected groups (G1 and G2) ($P < 0.01$) (Fig. 4) (Supplementary file 2).

3.6. Humoral immune responses of foetuses/lambs and parasite detection, parasite load and histopathology in placental tissues and foetal brain

All aborted foetuses from the infected groups were seropositive. IFAT titres ranged from 1:16 to 1:1024 (median IFAT titre of 1:256) in G1 (infected/treated) and from 1:128 to 1:1024 (median IFAT titre of 1:512) in G2 (infected/untreated). Significantly lower IFAT titres in aborted foetuses were found in G1 (infected/treated) than in G2 (infected/untreated) ($P < 0.05$). Additionally, lambs born from both infected groups were seropositive with IFAT titres of 1:1600 and 1:3200 and without significant differences between them (Supplementary file 2). Anti-*N. caninum* IgGs were not detected in the precolostral serum samples from lambs born in the uninfected group (G3).

Parasite DNA was found in all the placentas that could be collected from infected sheep. Ninety-five percent (40/42) and ninety-seven percent (41/42) of the placentome samples from aborted sheep in G1 (infected/treated) and G2 (infected/untreated) were PCR positive, respectively, and no significant differences were observed between them. No significant differences were observed in the parasite burden in placentomes from aborted sheep in infected groups (Fig. 5). Multifocal to coalescent necrotic placentitis, consistent with protozoal infection, was observed in the placentomes from all infected sheep, treated and not treated, where the placenta could be evaluated. These lesions were characterized by one (Fig. 6A) or several (Fig. 6B) foci of necrosis in the interdigitate area of the placentome with scant infiltration of mononuclear inflammatory cells. In the placentome showing more severe lesions, the necrotic foci converged, usually bridging the maternal end stalk of the placentome with the foetal chorionic plate. Concerning lesion scoring of the necrotic foci found in the placentomes, no significant differences were found between infected groups in the number of the lesions, average area of the lesions and the percentages of damaged area (Fig. 7). Regarding cotyledons from infected sheep that gave birth, 100% (6/6) of the samples from G1 (infected/treated) were PCR positive; however, no cotyledons could be recovered from the sheep that gave birth in G2 (infected/untreated). All cotyledons from G3 (uninfected/untreated) were PCR negative.

In foetal brains, 75% (27/36) and 77% (35/45) of the samples from G1 (infected/treated) and G2 (infected/untreated) were PCR positive, respectively, with no significant differences between them

(Supplementary file 2). In aborted foetuses, 83% (25/30) and 82% (32/39) of brain samples from G1 (infected/treated) and G2 (infected/untreated) were PCR positive, respectively. No significant differences were found in the brain parasite burden of aborted foetuses between the infected groups (Fig. 5). In lambs born, 33% (2/6) and 50% (3/6) of brain samples from G1 (infected/treated) and G2 (infected/untreated) were PCR positive, respectively. In both infected groups, lower brain parasite loads were found in lambs born than in aborted foetuses ($P < 0.05$). All brains from lambs born in G3 (uninfected/untreated) were PCR negative. Lesions were found in 75% (9/12) and 93% (14/15) of foetal brains in G1 (infected/treated) and G2 (infected/untreated), respectively, with no significant differences between them (Supplementary file 2). In aborted foetuses, 70% (7/10) and 100% (13/13) of brains showed non-purulent multifocal encephalitis in G1 (infected/treated) and G2 (infected/untreated), respectively. The affected brains showed diffuse congestion and haemorrhages, mainly in the white matter (Fig. 6C). In addition, glial foci were randomly distributed throughout the brain, variable in number and characterized by a central area of necrosis and peripheral infiltration of mononuclear cells (Fig. 6D). There were no significant differences between infected groups regarding the number of the lesions, average area of the lesions or the percentages of damaged area found in brains from aborted foetuses (Fig. 7). The percentage of lambs showing lesions in the brain ranged from 50% (1/2) in G2 (infected/untreated) to 100% (2/2) in G1 (infected/treated). No lesions were found in the brains from lambs born in G3 (uninfected/untreated).

4. Discussion

To date, only one study had been carried out to report on the efficacy of a drug for neosporosis in pregnant ruminants. In that study, BKI-1553 had shown partial efficacy against abortion and a lack of protection against vertical transmission (Sánchez-Sánchez et al., 2018c). BKI-1294 showed better efficacy in a pregnant mouse model of neosporosis than BKI-1553 (Winzer et al., 2015; Müller et al., 2017) and proved to be highly effective against *T. gondii* in pregnant sheep (Sánchez-Sánchez et al., 2019). Thus, the efficacy of BKI-1294 in a refined experimental ovine model of *N. caninum* infection during pregnancy was evaluated.

BKI-1294 showed a high plasma clearance and a 30-fold lower bioavailability in mice than BKI-1553 (Vidadala et al., 2016), which could be related to the lower PPB in mice of BKI-1294 (85%) than BKI-1553 (93%) (Schaefer et al., 2016). Here, the lower than 80% PPB of BKI-1294 in sheep could also lead to an efficient and rapid plasma clearance of the compound. Oral administration of 100 mg/kg BKI-1294 to *N. caninum*-infected pregnant sheep resulted in maximum concentrations of 2–3 μM, with trough plasma concentrations of 0.7–1.4 μM at 48 h after each dose. Contrary to the pharmacokinetics of BKI-1294 in non-infected pregnant sheep (Sánchez-Sánchez et al., 2019), a very slight accumulation in the plasma was found in this study, mainly after the last two doses, probably due to the saturation of hepatic metabolism of BKI-1294 with repeated administration as previously described (Ojo et al., 2013). In this study, there was a period of 12–15 days in which BKI-1294 plasma concentrations were above the IC_{50} for *N. caninum*. However, in the previous study on BKI-1553 treatment in pregnant sheep (Sánchez-Sánchez et al., 2018c), the period with plasma concentrations above the IC_{50} was much longer (at least 28–33 days).

An *in vitro* study of BKI-1294 using Nc-Spain7 isolate determined an IC_{50} of 0.27 ± 0.02 μM, albeit the IC_{50} differed between different *N. caninum* isolates (Winzer et al., 2015). Although drug plasma concentrations are higher than the IC_{50} for *N. caninum*, the time span during which plasma concentrations are above the IC_{50}, the PPB, and the penetration of the drug through the placental and blood-brain barriers could be crucial parameters that influence the efficacy of BKI-1294 in the refined ovine model of *N. caninum* infection during pregnancy. Furthermore, it is unknown how C_{max} and trough plasma concentrations in the dams (much higher than the IC_{50} for BKI-1553 in pregnant sheep
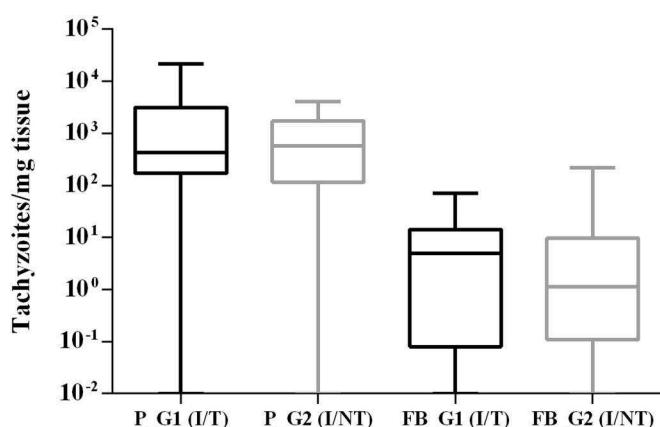

Fig. 5. Box-plot graphs of *N. caninum* burdens in placentomes (P) and foetal brain (FB) from aborted sheep in the infected groups. Graph represents the median percentage, the lower and upper quartiles (boxes) and minimum and maximum values (whiskers) for each organ in G1 (I/T) and G2 (I/NT). Considering that the *N. caninum* detection limit by real-time PCR is 0.1 parasites, negative samples (0 parasites) were represented on the log scale as<0.1 (i.e., 10^{-2}). Parasite burdens were analysed using the Mann–Whitney test. I/T means infected/treated and I/NT means infected/non-treated.
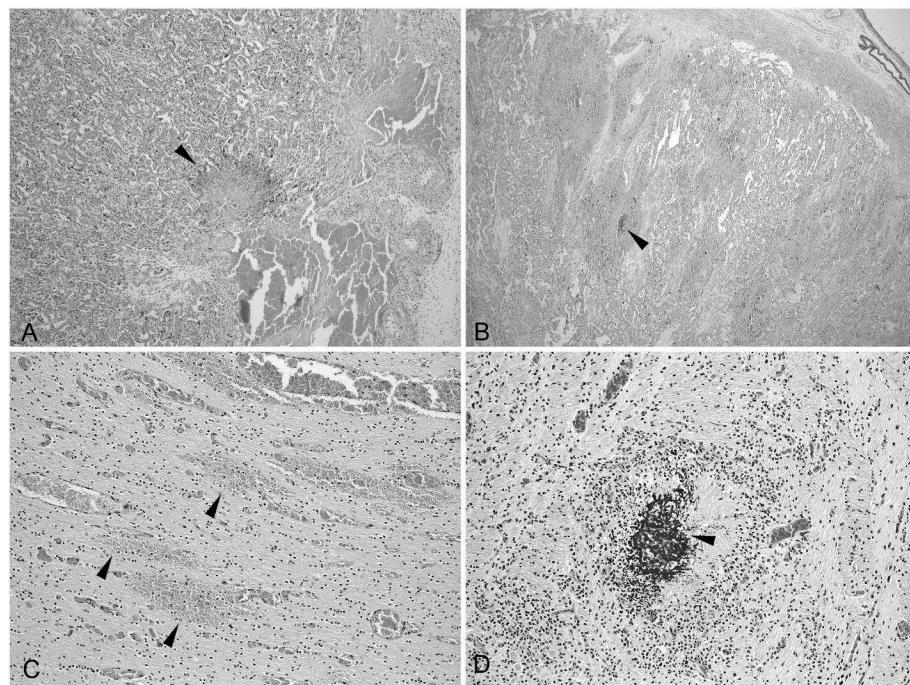
Fig. 6. Histological lesions observed in placentomes and foetal brain. Placentome. A sole focus of necrosis (arrowhead) at the interdigitate area close to the haemorrhage and the chorionic plate. HE, 40× (A). Placentome showing multifocal to coalescent placentitis. Some of the necrotic foci show mineralization at the centre of the necrotic area (arrowhead). HE, 12× (B). Foetal brain. General congestion and haemorrages (arrowheads) within the white matter. HE, 100× (C). Foetal brain. Glial foci with central areas of necrosis and mineralization (arrowhead) and peripheral infiltration of mononuclear cells. HE, 100× (D).

(Sánchez-Sánchez et al., 2018c) and only moderately higher than the IC_{50} for BKI-1294) could influence the efficacy in that model.

Here we followed an identical oral dosing regimen of BKI-1294 in *N. caninum*-infected pregnant sheep as was used in the pregnant sheep *T. gondii* model (Sánchez-Sánchez et al., 2019). Similar to that treatment, the compound was safe in this study, without an increase in rectal temperature or abortions associated with the drug application (both of which were associated with *N. caninum* infection since they occurred simultaneously in both infected groups, treated and untreated) or changes in behaviour and colour and consistency of faeces. Therefore, in *N. caninum*-infected sheep, the safety of oral administration of BKI-1294 was much better than that of subcutaneous administration of BKI-1553 (increase in rectal temperature, formation of transient dermal nodules and monocytosis) (Sánchez-Sánchez et al., 2018c).

To allow BKI-1294 a better chance to be effective than in the treatment of pregnant sheep with BKI-1553 (Sánchez-Sánchez et al., 2018c), a refined abortion model of infection at mid-pregnancy was employed, with a tenfold reduction of the intravenous dose of Nc-Spain7 tachyzoites (Sánchez-Sánchez et al., 2018a). In addition, to ensure that efficacy results were comparable to previous BKI therapy of neosporosis in mice (Winzer et al., 2015; Müller et al., 2017) and sheep (Sánchez-Sánchez et al., 2018c), BKI-1294 treatment was also started at 48 h pi. Rectal temperatures in the *N. caninum*-infected/untreated group increased from day 5 to day 8 pi, similar to previously reported results using the refined ovine model of infection, as a result of the first cycles of parasite replication (Sánchez-Sánchez et al., 2018a). Comparing both infected groups, a significantly lower increase in rectal temperatures was observed on days 5, 7, and 9 pi in the infected and BKI-1294-treated group than in the infected but untreated group. These differences between the treated and the untreated group could indicate an initially decreased parasite replication, and they were more pronounced than the differences observed after treatment with BKI-1553, where they did not exist or were more sporadic (Sánchez-Sánchez et al., 2018c). In addition, BKI-1294 treatment led to a dose-response effect (statistically lower rectal temperatures were found one day after the second, third and fourth doses, matching the drug peaks) similar to what was found after BKI-1294 treatment of *T. gondii*-infected sheep (Sánchez-Sánchez et al., 2019). This dose-response effect might indicate that the drug was effective early after infection; however, the lack of significant

differences in rectal temperatures on consecutive days indicates that following an initial attenuation, the parasite resumed replication.

Several reports have shown that IFNγ release early after *N. caninum* infection is essential to develop host protective immunity (Innes and Mattsson 2007; Mineo et al., 2010). In this study, the infected/untreated group showed an increase in IFNγ release in stimulated peripheral blood cultures at day 7 pi, similar to the IFNγ kinetics in the experimental model of *N. caninum* infection used here (Sánchez-Sánchez et al., 2018a). When analysing the effect of BKI-1294 treatment, significantly higher IFNγ levels were found on day 7 pi in the infected/treated group compared to the infected/non-treated group, similar to what was previously described after treatment with BKI-1553 of *N. caninum*-infected pregnant sheep (Sánchez-Sánchez et al., 2018c). Additionally, although not statistically significant, 10-fold higher IFNγ release on days 14, 21, and 28 pi was found in the infected/treated group than in the infected/untreated group in this study. Differences in IFNγ levels beyond day 7 pi have also been described after the treatment of *N. caninum*-infected pregnant sheep with 7 doses (but not after 2 doses) of BKI-1553 (Sánchez-Sánchez et al., 2018c); therefore, long-term treatment with these BKIs seems to be needed to maintain higher IFNγ levels compared to infected/untreated groups. These increased levels of IFNγ in treated animals might have led to greater initial control of parasitaemia (Entrican 2002; Innes 2007). BKI-1294, similar to BKI-1553, exhibited a parasitostatic effect *in vitro* and the formation of intracellular multinucleated complexes since these drugs blocked the egress of tachyzoites, but the new forms re-established replication after removal of the drug (Winzer et al., 2015; Müller et al., 2017). *In vivo*, these intracellular complexes might not be able to escape from the immune system, being more exposed to antigen-presenting cells and therefore this could result in higher IFNγ levels in treated animals.

Following the time course of assessments after *N. caninum* infection, IgG levels against *N. caninum* soluble antigens in the dams from the infected/untreated group began to rise on day 14 pi, in line with anti-*N. caninum* IgG kinetics previously found in the experimental model of *N. caninum* infection used here (Sánchez-Sánchez et al., 2018a). On the other hand, the infected/treated group showed lower anti-*N. caninum* IgG levels against *N. caninum* soluble antigens on days 14, 21, and 28 pi compared to the infected/untreated group, likely due to lower early antigen stimulation. In the previous BKI-1553 efficacy study in

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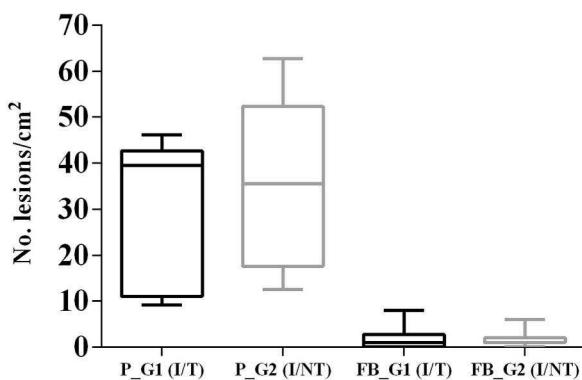
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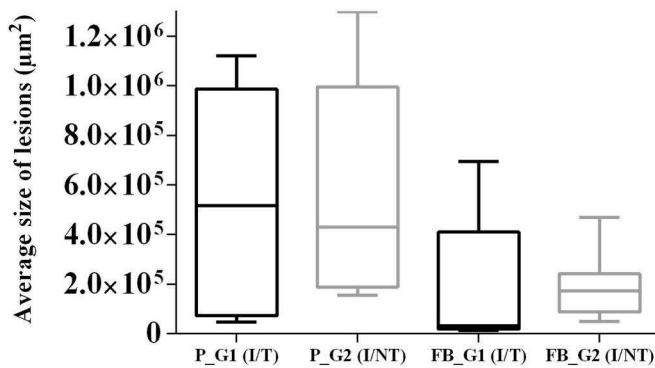
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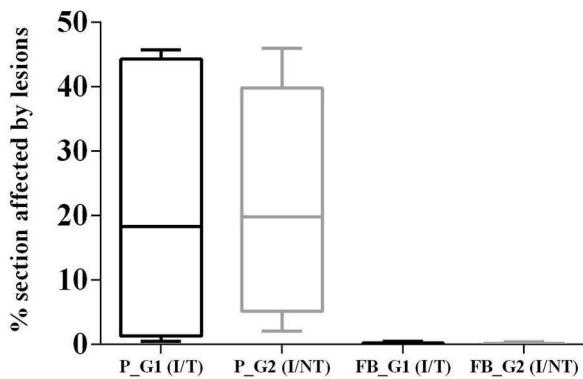
Fig. 7. Box plots showing the number of lesions (A), average size of the lesions (B) and lesion rates (C) in placentomes (P) foetal brain (FB) from aborted sheep in the infected groups. Graphs represent the median percentage, the lower and upper quartiles (boxes) and minimum and maximum values (whiskers) for each organ in G1 (I/T) and G2 (I/NT). Histological measurements of lesions were compared using the Mann–Whitney test. I/T means infected/treated and I/NT means infected/non-treated.

N. caninum-infected pregnant sheep, lower antibody levels associated with drug administration were described from day 21 pi until the end of the experiment (49 days pi) (Sánchez-Sánchez et al., 2018c). However, these differences in anti-*N. caninum* IgG levels against *N. caninum*

soluble antigens associated with BKI-1294 treatment were not found from day 35 pi onwards, probably because the time span when plasma concentrations were above the IC_{50} for BKI-1294 was more than 2 times shorter than for BKI-1553. Consequently, after the short plasma exposure for the parasitostatic BKI-1294, the parasite recommenced proliferation and antibody levels increased.

Recently cross reactions between TgSAG1 and TgSAG1-GRA8 chimeric antigen and *N. caninum* had been detected using sheep sera (Huertas-López et al., 2021). In this study, also cross reaction was found between TgSAG1 and *N. caninum*, and although more studies are needed (e.g., test *N. caninum*-positive sera from other hosts), the use of the commercial kit "ID Screen® toxoplasmosis indirect multispecies", using TgSAG1, could prompt false positive results for *T. gondii* due to cross reactions with *N. caninum*. We aimed to investigate anti-NcSAG1 and anti-NcSAG4 IgGs in dams. Previous studies showed that immunofluorescence staining of NcSAG1, a tachyzoite antigen, during *in vitro* treatment with BKI-1294 was diminished in the multinucleated complexes; however, 10 days after drug removal, the newly formed tachyzoites showed well-defined NcSAG1 staining (Winzer et al., 2020a). Analysing the results derived from BKI-1294 treatment, there were lower anti-NcSAG1 antibodies in treated animals on day 21 pi because during BKI-1294 treatment, there was a lower presence of NcSAG1 in the multinucleated complexes and therefore lower exposure to the immune system. However, the significant differences in anti-NcSAG1 antibodies between the treated and untreated animals decreased on day 42 pi, likely due to reactivation of the infection with an increased presence of tachyzoites after drug clearance.

In the proteome of the multinucleated complexes formed after *in vitro* treatment of *N. caninum* with BKI-1294, the expression of NcSAG4, a bradyzoite antigen, was the most upregulated in these multinucleated complexes (Winzer et al., 2020b). In this study, the recognition of NcSAG4 by WB was higher in untreated dams than in treated dams on days 21 and 42 pi due to a likely higher presence of bradyzoites in tissue cysts in the untreated dams. The weak to moderate recognition of NcSAG4 on days 21 and 42 pi found in the treated dams could be due to the presence of multinucleated complexes and/or to the partial efficacy of BKI-1294 against infection in the dams.

The abortion and vertical transmission rates described here are in agreement with those described in the refined experimental abortion model employed (Sánchez-Sánchez et al., 2018a). Thus, similar to the 100% abortion rate previously reported, in this study, only one out of eight infected/untreated ewes gave birth, and this difference could be due to the larger number of animals used. Seroconversion in 100% of foetuses or lambs, more than 77% parasite detection in the foetal brain and more than 93% of foetuses/lambs with brain lesions were similarly described. BKI-1294 treatment did not prevent abortion at all (the same number of ewes aborted in the treated group and the untreated group), obtaining worse protection against abortion in sheep compared with the use of BKI-1553, which allowed 37–50% of ewes to give birth (Sánchez-Sánchez et al., 2018c). BKI-1294 treatment only slightly reduced the IgG response in the aborted foetuses, likely due to a lower antigenic stimulation of the foetuses from treated ewes early after infection, but did not prevent vertical transmission or reduce parasite detection, parasite load or lesions in the foetal brain. The lack of differences between infected groups in parasite detection, parasite load and lesions in the foetal brain, along with abortions at the same rate and at the same time in both groups, could indicate that therapeutic concentrations of BKI-1294 could not be reached in the foetal brain, or only for insufficient durations. If the 20–30% placental crossing of BKI-1553 (Sánchez-Sánchez et al., 2018c) is similar to the placental crossing of BKI-1294 and if the 30% CNS crossing of BKI-1294 in mice (Vidadala et al., 2016) is also true in sheep, at the troughs, concentrations in the foetal brain would be below the IC50 for *N. caninum*. The effect of BKI-1294 on vertical transmission was even worse than the effect of BKI-1553 on vertical transmission, which did not avoid vertical transmission but, although tested in a more aggressive model of infection,

reduced parasite detection by 25% and parasite load by 6 times in the foetal brain (Sánchez-Sánchez et al., 2018c). The absence of protection of BKI-1294 against abortion and vertical transmission in sheep infected with *N. caninum* at mid-pregnancy contrasts starkly with the 87% protection against pup mortality and the 84% protection against vertical transmission found in a pregnant mouse model of neosporosis after treatment with this compound (Winzer et al., 2015), which could be because: 1) intravenous inoculation used here was more aggressive than subcutaneous infection in mice, 2) there was only a 6-day interval between the end of the BKI-1294 parasitostatic treatment and delivery in mice, while in sheep, there was a 50-day interval, allowing extensive parasite replication in sheep after drug clearance, 3) the higher number of layers between maternal and foetal blood in sheep than in mice (Walker et al., 2017), although not studied, could hamper the placental transfer of BKI-1294 in sheep, and 4) contrary to mice, the lack of maternal antibodies crossing the placental barrier in sheep might have favoured higher parasite replication in ovine foetuses. The absence of protection of BKI-1294 against abortion and vertical transmission in sheep infected with *N. caninum* tachyzoites at mid-pregnancy also differs sharply from the high efficacy found in sheep infected with *T. gondii* oocysts at mid-pregnancy (Sánchez-Sánchez et al., 2019). This could be due to the fact that at the beginning of the treatment (48 h post-infection), *T. gondii* had not yet reached the foetus (Sánchez-Sánchez et al., 2019), while in this experiment intravenously inoculated *N. caninum* tachyzoites would probably already be replicating in the foetal tissues.

The results reported here indicate that BKI-1294 treatment of *N. caninum*-infected sheep at mid-pregnancy caused a decrease in rectal temperatures, an increase in peripheral IFNγ levels and a transient decrease in IgG levels against *N. caninum* soluble antigens in the dams. Additionally, the presence of anti-NcSAG1 and anti-NcSAG4 IgGs was lower in treated dams on days 21 and 42 pi. However, a short systemic exposure (and probably insufficient foetal exposure) to this parasitostatic drug resulted in a lack of foetal protection. Therefore, future studies on the efficacy of BKIs against *N. caninum* infection in ruminants experimentally infected at mid-pregnancy should be extended in the time and carried out with BKIs showing a very low plasma clearance and good ability to cross the placental and blood-brain barriers. Additionally, the absorption of BKIs after oral administration should be improved. With regard to the ovine models of neosporosis to test novel BKIs, an experimental abortion model after subcutaneous infection in sheep at mid-pregnancy with 10^4 Nc-Spain7 tachyzoites, closer to natural conditions than intravenous infection, could be used (Sánchez-Sánchez et al., 2018a). Once promising results are obtained in an improved model of infection, the efficacy of BKIs could be tested against the recently described highly efficient endogenous transplacental transmission of *N. caninum* in naturally infected sheep (González-Warleta et al., 2018) and against exogenous and endogenous transplacental transmission in cattle.

Author contributions

IF, AH, WVV and LMO proposed and designed the study. RSS wrote the manuscript, interpreted the results and was helped by IF, GAG, JB, AH, WVV and LMO in the discussion. LMF and HR selected and cared for the animals and carried out oestrus synchronization and mating. RSS, IF and MR prepared the N. caninum doses and performed the infections. RSS, IF, MR, BPA, DCR, VHG and MPD participated in clinical assessment and blood sampling and collected post mortem samples. KO, MAH, RC, and GRW determined the pharmacokinetics of the compound. RSS performed peripheral blood stimulation assays, ELISA, PCR and qPCR assays, statistical analysis and interpreted the results. RVG and JB carried out histological examination. All authors read and approved the final manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpddr.2021.10.001.

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